"DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL BUTYROLACTONE DERIVATIVES OF CHALCONE AS ANTITUBERCULAR AGENTS"

A dissertation submitted to

THE TAMIL NADU Dr.M.G.R MEDICAL UNIVERSITY
CHENNAI- 600 032

In partial fulfillment of the requirements
for the award of the degree of

MASTER OF PHARMACY

in

PHARMACEUTICAL CHEMISTRY

Submitted by

261215708

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

COLLEGE OF PHARMACY

MADRAS MEDICAL COLLEGE

Chennai- 600 003

April 2014

ACKNOWLEDGEMENT

First and foremost I thank the almighty for all the love, grace and blessings he has bestowed upon me and for strengthening me throughout to successfully complete this dissertation.

I take this opportunity to express my gratitude to the people who were instrumental in the successful completion of this project.

At this moment of accomplishment, first of all I express my deep sense of gratitude to Mrs.Vimala M.D., Dean, Madras Medical College, Chennai- 3 for providing all facilities during the period of study.

It is my privilege and great pleasure to express my gratitude to my esteemed Principal and Guide Dr.A.Jerad Suresh, M.Pharm., Ph.D., M.B.A., College of Pharmacy, Madras Medical College, Chennai-3.This work would not have been possible without his able guidance, support and encouragement. Under his guidance I successfully overcame many difficulties and learned a lot. His passion for perfection combined with eagerness and enthusiasm to impart knowledge to students is what I am in awe of. His knowledge and commitment to the highest standards inspired and motivated me. I immensely thank him for his guidance.

I take this opportunity to sincerely thank Dr.V.Niraimathi, M.Pharm., Ph.D., Assistant Reader in Pharmacy, Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai-3 for her support and encouragement towards the successful completion of this dissertation.

I heartily thank Mrs.R.Priyadharsini M.Pharm., Mrs.P.G.Sunitha M.Pharm., Mrs.T.Saraswathy M.Pharm., Dr.M.Sathish M.Pharm., Ph.D., for the help they rendered throughout this study.

I am extremely thankful to Mr.D.Sivakumar, Lab Supervisor, Department of Pharmaceutical Chemistry for his constant support during my experimentation. I also thank Mr.Bhaskar, Lab Supervisor for his help.

I thank Mrs.Bhooma, Mrs.Maheswari, Mrs.Murugeshwari, Mrs.Geetha, Mrs.Mala, Lab technicians, College of Pharmacy for their support.

I thank Mr.K.M.Noorulla M.Pharm. (Ph.D), Mrs. Devi Umesh, M.Pharm. (Ph.D) Ms.P.R.Suriya, M.Pharm. (Ph.D), Ph.D Scholars for their constant support, valuable suggestions and comments during my research.

My heartfelt thanks to CARISM, SASTRA University, Tanjavur, Dept of Chemistry, IIT Madras and SAIF, IIT Madras for helping me to carry out the characterization studies.

I express sincere thanks to Dr.Kishore Bhat, Principal, Department of Microbiology, Martha Mandal's NGH Institute of Dental Sciences and Research Centre, Belgaum for helping me to carry out the in vitro anti tubercular activity.

A heartful thanks to Sathya Sukanya for her care, constant help, support and motivation during the entire period. My heartfelt thanks to my friends Jennifer and Kishore for their care, support and encouragement throughout. I wish to thank my sisters Rama Prabha and Maheswari for their care, timely help and moral support. I heartily thank Vengatesh for his constant support. I thank all my friends Bakkiyaraj, Kumar, Mahesh, Murugan and Senthil for all the help they rendered.

My special thanks to Satheesh Kumar for his guidance to start this project. I take immense pleasure in thanking all my seniors, juniors and friends from all the other departments.

Finally yet importantly, I love to express my heartfelt thanks to my beloved parents, brother for their blessings, encouragement and wishes for the successful completion of this dissertation.

CONTENTS

LIST OF ABBREVIATIONS USED

TB	Tubercle Bacillus
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacille Calmette Guerin
DOT	Directly Observed Therapy
MDR-TB	Multidrug-resistant TB
XDR-TB	Extensively drug-resistant TB
LTBI	Latent Tuberculosis Infection
LdtMt2	Mycobacterium L, D-transpeptidases 2
CADD	Computer-Aided Drug Design
SAR	Structure-Activity Relationship
QSAR	Quantitative Structure-Activity Relationship
ADME	Absorption, Distribution, Metabolism and Excretion
PSA	Polar Surface Area
OSIRIS	Optical, Spectroscopic and Infrared Remote Imaging System
GLIDE	Grid Based Ligand Docking With Energetics
G Score	Glide Score
OPLS	Optimized Potential for Liquid Simulations
TPSA	Total Polar Surface Area
Log P	Partition Co-Efficient
WHO	World Health Organization
MIC	Minimum Inhibitory Concentration
PDB	Protein Data Bank
TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
IR	Infrared Spectroscopy
REMA	Resazurin Micro Plate Assay
NRA	Nitrate Reductase Assay
MABA	Micro Plate Alamar Blue Assay

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MADRAS MEDICAL COLLEGE
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TAMILNADU

CERTIFICATE

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Dr.A.Jerad Suresh, M.Pharm., Ph.D., M.B.A
Principal,
College of Pharmacy,
Madras Medical College,
Chennai-600003

Place: Chennai
Date:

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
COLLEGE OF PHARMACY
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Dr.A.Jerad Suresh, M.Pharm., Ph.D., M.B.A
Principal,
College of Pharmacy,
Madras Medical College,
Chennai-600003

Place: Chennai
Date:

Dedicated to the
Almighty
& my Family

Introduction

INTRODUCTION

Mycobacterium tuberculosis is the bacteria that cause tuberculosis (TB). It has been present in the human population since antiquity - fragments of the spinal column from Egyptian mummies from 2400 BC show definite signs of tuberculosis. In 1882, Robert Koch discovered a staining technique that enabled him to see Mycobacterium tuberculosis. Tuberculosis is contagious and airborne. It ranks as the second leading cause of death from a single infectious agent, after the human immunodeficiency virus (HIV).[1]

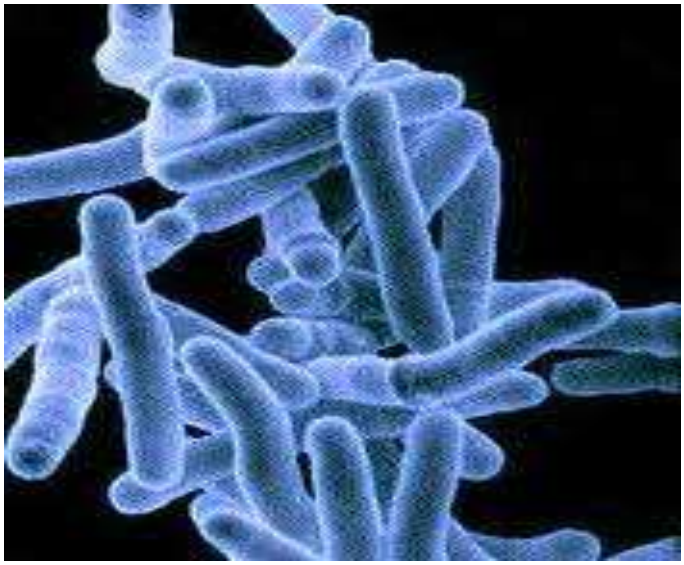


Fig.1: Electron microscope photograph of Mycobacterium tuberculosis.

Tubercle bacilli are the rod-shaped, which are spread mostly through air-born droplets or dust micro-particles of dried sputum. Once inhaled, the immune system reacts by engulfing the bacteria, forming a tubercle which prevents it from spreading.

In most cases, the bacteria will die. If it survives, it becomes dormant and the infected individual may develop active disease at a later date, sometimes soon after infection, sometimes years later.

Those who develop active pulmonary tuberculosis experience a range of signs and symptoms, including chest pain, cough, weight loss, pallor, fever, and night sweats. People with suppressed immune systems, such as persons with HIV and AIDS, are prone to develop active tuberculosis. Only those with active TB are able to spread it, by coughing or sneezing. These actions release the bacteria from the lungs into the air, from where it can be inhaled by others. The bacteria are able to survive in the air for several hours, but are weakened by direct sunlight.[2]

PATHOPHYSIOLOGY

Humans are the only known reservoir for *Mycobacterium tuberculosis*. TB is transmitted by airborne droplet nuclei, which may contain fewer than 10 bacilli. TB exposure occurs by sharing common airspace with an individual who is in the infectious stage of TB.

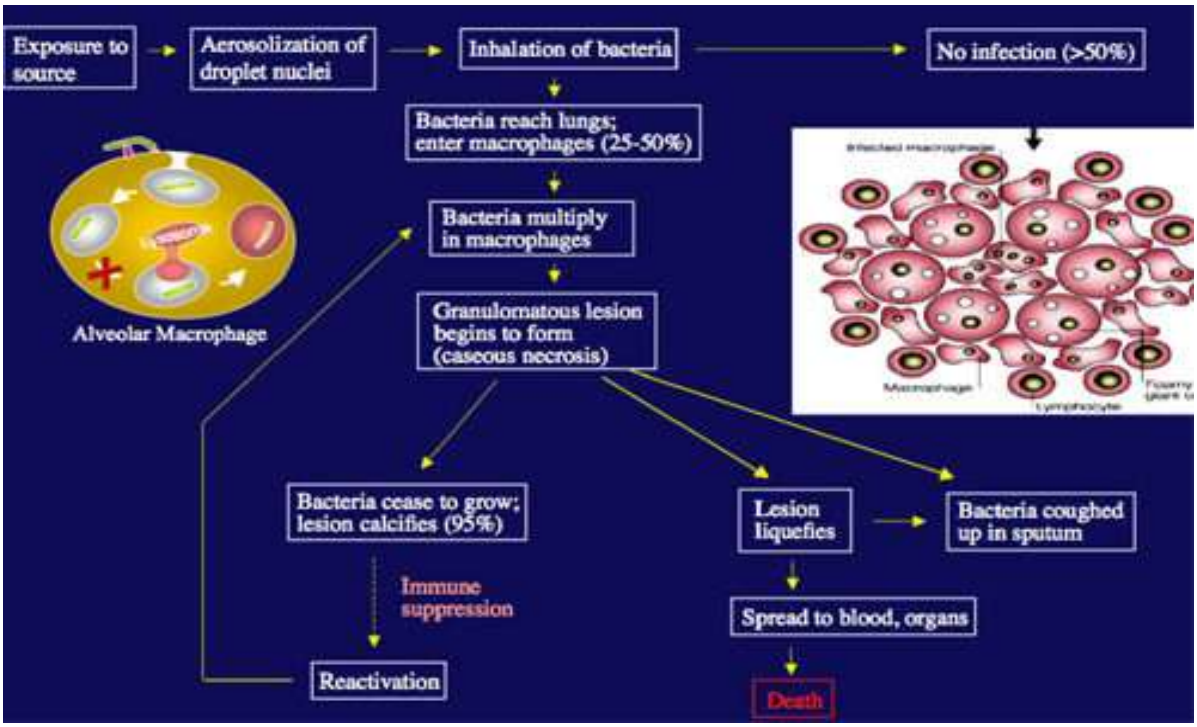


Fig.2: Pathogenesis of Mycobacterium tuberculosis.

1. Inhalation

The bacteria are inhaled. The majority of the bacteria will become lodged in the upper respiratory tract, namely the nose and throat, where their survival is difficult. Some of the smaller particles, though, will make it into the lungs and alveoli where infection sets in. Alternatively, the bacteria might be ingested.[3]

2. Bacteria multiplication

In the alveoli, the bacteria are engulfed by inactivated macrophages, white blood cells present within tissues, where they multiply until the macrophage bursts. The Mycobacterium tuberculosis replicates very slowly, only once every twenty four hours, and takes up to one month to form a colony.

3. T-cell activation

Dendritic cells are a key part of the mammalian immune system. When dendritic cells detect foreign substances entering the body, they engulf and bring them to the lymph nodes where they present the antigens to certain white blood cells called T-cells. If the T cell has a specific receptor for the presented antigen it will become activated to release potent molecules, such as interferon-gamma and tumour necrosis factor, which in turn stimulate macrophages and other T cells to produce a cell-mediated response against the bacteria carrying those antigens.

4. Tubercle Formation

The T-cells return to the site of infection through the blood stream, where they contribute to the formation of a tubercle or granuloma. The TB tubercle is made up of a core of infected macrophages, a surrounding ring of foamy macrophages and an outside ring of T-cells, all enveloped in a fibrous shell. In some cases, an individual's immune system is unable to defend against the bacteria by creating a tubercle to isolate it. Primary progressive tuberculosis occurs as a result. This is mostly seen in young children or individuals with much suppressed immune systems.

When contained inside the granuloma the bacteria are inactive and the case of tuberculosis is considered to be latent. The bacteria are contained in the granuloma until the immune system is weakened, breaking down the outer ring of the tubercle, releasing the bacteria inside. In this situation, the case of tuberculosis has been reactivated and is known as secondary progressive tuberculosis. Only approximately 3-5% of immuno-competent individuals will develop secondary progressive tuberculosis within two years of the primary infection, and a further 3-5% will develop it after two years.[4]

5. Cavitation and Tubercle break-down

In some cases, the damaged cells at the centre of the granuloma liquefy. The bacteria grow well in this liquid, multiplying outside of macrophages, their typical hosts. As they multiply, the tubercle enlarges. This can cause nearby tissue in the lungs to die and rupture, forming a cavity, or the tubercle to burst spreading bacteria further around the lungs or the body. This would also be considered a case of secondary progressive tuberculosis. The immune system will respond as per steps 3 & 4 when the bacteria are recognized in their new locations.

A small number of tubercle bacilli enter the bloodstream and spread throughout the body. The tubercle bacilli may reach any part of the body, including areas where TB disease is more likely to develop (such as the brain, larynx, lymph node, lung, spine, bone, or kidney).[5]

EPIDEMIOLOGY

The highest incidences are seen in those countries of Africa, Asia, and Latin America. The World Health Organization estimates that nine million people get TB every year, of whom 95% live in developing countries. An estimated 2-3 million people die from TB every year.

8.6 million People fell ill with TB in 2012, including 1.1 million cases among people living with HIV. In 2012, 1.3 million people died from TB, including 320 000 among people who were HIV-positive. The TB mortality rate has decreased 45% since 1990, and the 2015 global target of a 50% reduction in mortality is now within reach.[6]

RISK FACTORS

- Working in the health care profession or as an embalmer
- Being born in or spending time in a country where TB is common (for instance, most countries in Latin America and the Caribbean, Africa, and Asia, excluding Japan)
- Living in overcrowded areas
- Unsanitary settings where TB is common (for example, homeless shelters, migrant farm camps, prisons and jails, and some nursing homes or long-term care facilities)
- Having HIV or AIDS. As HIV attacks the immune system, existing TB infections may become active, or it may make it easier for someone to catch TB. The TB bacteria, in turn, cause the HIV virus to replicate more quickly.
- Using medications that suppress the immune system
- Smoking and alcoholism
- Organ transplantation
- Having no or inadequate access to health care Having diabetes (the risk of contracting TB is 2-3 times higher among people who have diabetes compared to people who don't have diabetes)
- Having a rheumatic disease

TUBERCULOSIS SIGNS AND SYMPTOMS

- Mild fever
- Headache
- Chills, Night sweats
- Fatigue
- Loss of appetite, weight loss
- Cough with or without mucus and pus
- Coughing up blood
- Chest pain from inflammation in the lungs
- Difficulty breathing
- Sore throat.[7]

MEDICAL CARE

The tuberculosis vaccine, known as bacille Calmette-Guerin (BCG) may prevent the spread of tuberculosis and tuberculous meningitis in children, but the vaccine does not necessarily protect against pulmonary tuberculosis.

The chemotherapy of infectious diseases, using sulfonamide and penicillins, had been underway for several years, but these molecules were ineffective against Mycobacterium tuberculosis. From 1943 Streptomycin was used in treatment. Following streptomycin, p-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962), and rifampin (rifampicin; 1963) were introduced as anti-TB agents.The initial combination of isoniazid, rifampin, pyrazinamide, and ethambutal provide effective TB treatment.[8]

DIRECTLY OBSERVED THERAPY

For initial empiric treatment of tuberculosis (TB), start patients on a 4-drug regimen: isoniazid, rifampin, pyrazinamide, and either ethambutol or streptomycin. Once the TB isolate is known to be fully susceptible, ethambutol (or streptomycin if used as a fourth drug) can be discontinued.

After 2 months of therapy (for a fully susceptible isolate), pyrazinamide can be stopped. Isoniazid plus rifampin are continued as daily or intermittent therapy for 4 more months. If isolated isoniazid resistance is documented, discontinue isoniazid and continue treatment with rifampin, pyrazinamide, and ethambutol for the entire 6 months. Therapy must be extended if the patient has cavitary disease or remains culture-positive after 2 months of treatment.[9]

Directly observed therapy (DOT) is recommended for all patients. Patients on the above regimens as DOT can be switched to 2- to 3-times per week dosing after an initial 2 weeks of daily dosing. Patients on twice-weekly dosing must not miss any doses. Prescribe daily therapy for patients on self-administered medication.[10]

DRUG RESISTANCE

TB organisms resistant to the antibiotics used in its treatment are widespread and occur in all countries. Drug resistance emerges as a result of inadequate treatment and once TB organisms acquire resistance they can spread from person to person in the same way as drug-sensitive TB.

MDR-TB

Multidrug-resistant TB (MDR-TB) is caused by organisms that are resistant to the most effective anti-TB drugs (isoniazid and rifampicin). MDR-TB results from either infection with organisms which are already drug-resistant or may develop in the course of a patient's treatment.[11]

XDR-TB

Extensively drug-resistant TB (XDR-TB) is a form of TB caused by organisms that are resistant to isoniazid and rifampicin (i.e. MDR-TB) as well as any fluoroquinolone and any of the second–line anti-TB injectable drugs (amikacin, kanamycin or capreomycin).

These forms of TB do not respond to the standard six month treatment with first-line anti-TB drugs and can take two years or more to treat with drugs that are less potent, more toxic and much more expensive.[12]

THE NEED FOR NEW TUBERCULOSIS DRUGS

There are reasons usually given for the need of new tuberculosis drugs:

- To shorten the total duration of treatment.
- To prevent complications and development of latency
- To prevent subsequent recurrences
- To improve the treatment of MDR- TB
- To provide more effective treatment for latent tuberculosis infection (LTBI)

- To reduce both the total length of treatment and the frequency of drug administration.

CELL WALL OF MYCOBACTERIUM TUBERCULOSIS

Mycobacterium tuberculosis has a unique cell wall structure crucial for its survival. The well- developed cell wall contains a considerable amount of a fatty acid, mycolic acid, covalently attached to the underlying peptidoglycan bound polysaccharide arabinogalactan, providing an extraordinary lipid barrier. This barrier is responsible for many of the medically challenging physiological characteristics of tuberculosis, including resistance to antibiotics and host defense mechanisms.[13] The composition and quantity of the cell wall components affect the bacteria's virulence and growth rate. The peptidoglycan polymer confers cell wall rigidity and is just external to the bacterial cell membrane, another contributor to the permeability barrier of mycobacteria. Another important component of the cell wall is lipoarabinomannan, a carbohydrate structural antigen on the outside of the organism that is immunogenic and facilitates the survival of mycobacteria within macrophages.[14]

The cell wall is key to the survival of mycobacteria and a more complete understanding of the biosynthetic pathways, gene functions and the development of antibiotics to prevent formation of the cell wall are areas of great interest.

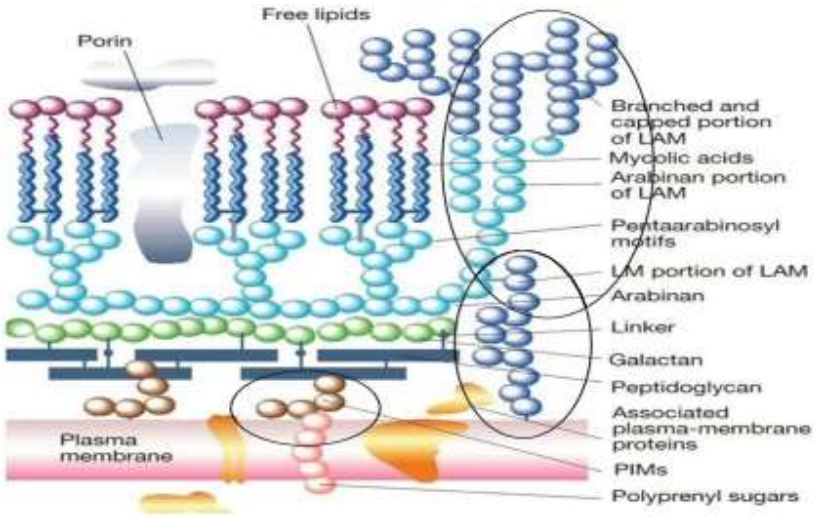


Fig.3: Cell wall structure of Mycobacterium tuberculosis.

TARGET: L, D-TRANSPEPTIDASE 2

With multi-drug resistant cases of tuberculosis increasing globally, better antibiotic drugs and novel drug-targets are becoming an urgent need. Traditional β-lactam antibiotics that disrupt the D, D-transpeptidases are not effective against mycobacteria, in part because mycobacteria rely mostly on β-lactam insensitive L, D-transpeptidases for biosynthesis and maintenance of their peptidoglycan layer.

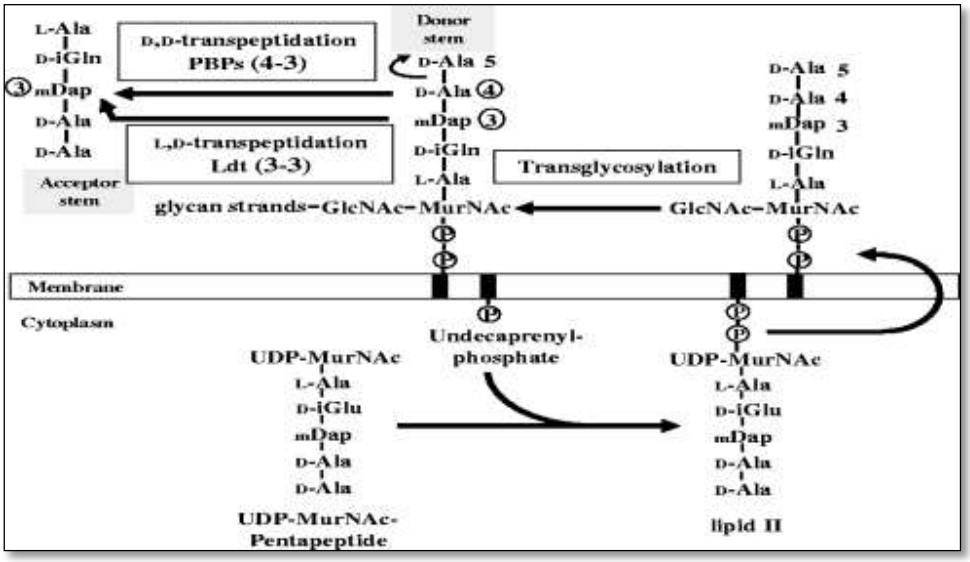


Fig.4: Role of L,D-transpeptidase in peptidoglycan synthesis.

This reliance plays a major role in drug-resistance and persistence of Mycobacterium tuberculosis infections. The crystal structure at 1.7 Å resolution of the Mycobacterium tuberculosis L,D-transpeptidase LdtMt2 containing a bound peptidoglycan fragment provides information about catalytic site organization as well as substrate recognition by the enzyme. Based on structural, kinetic, and calorimetric data, a catalytic mechanism for LdtMt2 in which both acyl-acceptor and acyl-donor substrates reach the catalytic site from the same, rather than different, entrances has been proposed by Sabri B. Erdemli et al. Together, this information provides vital insights for the development of novel drugs targeting this validated yet unexploited enzyme.[15]

MEDICINAL CHEMISTRY

Medicinal chemistry is best to be defined as an interdisciplinary research area incorporating different branches of chemistry and biology in the research for better and new drugs (Drug Discovery). In other words, medicinal chemistry is the science, which deals with the discovery and design of new and better therapeutic chemicals and development of these chemicals into therapeutic chemicals and development of these chemicals into new medicines and drugs.[16]

DRUG DISCOVERY, DESIGN AND DEVELOPMENT

The process of drug discovery is very complex and requires an interdisciplinary effort to design effective and commercially feasible drugs. The current scenario of development of new drugs needs no emphasis in light of the current global situation of health and disease. For the majority of time, drug discovery has been a trial-and-error process. Conventionally, the process of drug development has revolved around an almost blind screening approach, which was very time-consuming and laborious. The disadvantages of conventional drug discovery as well as the allure of a more deterministic approach to combat disease have led to the concept of "Rational drug design" in the 1960's. New understanding of the quantitative relationship between structure and biological activity ushered in the beginning of computer-aided drug design (CADD). With the introduction of integration and knowledge management solutions with the help of computers, a new era is beginning in drug discovery. The development cost will be cut by almost a third. The development times are reduced. At the onset, it is important to know what features an "ideal" drug should have. The drug

i. must be safe and effective
ii. should be well absorbed orally and bioavailable
iii. metabolically stable and with a long half-life
iv. nontoxic with minimal or no side effects
v. should have selective distribution to target tissue.[17]

Lead identification

A lead is defined as a compound (usually a small organic molecule) that demonstrates a desired biological activity on a validated molecular target. To fulfill the criteria of what the industry considers a useful lead, the compound must exceed a specific potency threshold against the target (e.g., < 10 μM inhibition). The compounds used as potential leads could come from many sources. A majority of leads discovered in very recent programs are derived from a collection that is now referred to as a "library". These may take the form of natural product libraries, peptides libraries, carbohydrates libraries, and/or small molecule libraries based on a variety of different molecular scaffolds.

Lead optimization

Once a lead compound is established in the identification process, the medicinal chemist will work closely with molecular pharmacologists to optimize the desirable traits of the lead. This process can be relatively fast since history has taught the medicinal chemistry community how to manipulate molecules to improve activity. Starting with intuitive structural modification to the development of structure-activity relationship (SAR) and quantitative SAR (QSAR) one can gain tremendous information.

COMPUTER-AIDED DRUG DESIGN

Computer-aided drug design uses computational chemistry to discover, enhance, or study drugs and related biologically active molecules. The most fundamental goal is to predict whether a given molecule will bind to a target and if so how strongly. Molecular mechanics or molecular dynamics are most often used to predict the conformation of the small molecule and to Molecular mechanics or molecular dynamics are most often used to predict the conformation of the small molecule and to model conformational changes in the biological target that may occur when the small molecule binds to it. This provides semi-quantitative prediction of the binding affinity. Also, knowledge-based scoring function may be used to provide binding affinity estimates. These methods use linear regression, machine learning, neural nets or other statistical

techniques to derive predictive binding affinity equations by fitting experimental affinities to computationally derived techniques to derive predictive binding affinity equations by fitting experimental affinities to computationally derived interaction energies between the small molecule and the target.[18]

RATIONAL DRUG DESIGN

Drug design is a process which involves the identification of a compound that displays a biological profile and ends when the biological profile and chemical synthesis of the new chemical entity are optimized. Drug designing is otherwise known as rational drug design and it is a method of finding new medications based on the biological receptors and target molecules. The objective of drug design is to find a chemical compound that can fit to a specific cavity on a protein target both geometrically and chemically.

TYPES OF DRUG DESIGN

- Ligand-based drug design
- Structure-based drug design

Ligand-based drug design:

Ligand-based drug design is an indirect approach which relies on knowledge of other molecules that bind to the biological target of interest. These other molecules may be used to derive a pharmacophore model that defines the minimum necessary structural characteristics a molecule must possess in order to bind to the target. In other words, a model of the biological target may be built based on the knowledge of what binds to it and this model in turn may be based on the knowledge of what binds to it, and this model in turn may be used to design new molecular entities that interact with the target.

Structure-based drug design:

Structure-based drug design is a direct approach which relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography or NMR spectroscopy. If an experimental structure of a target is not available it may be possible experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist or various automated computational procedures to suggest new drug candidates.[19]

DOCKING

Docking is simply referred to the ability to position a ligand in the active or a designated site of a protein and calculate specific binding affinities. Ligand-protein docking has evolved so remarkably throughout the past decade that docking single or multiple small molecules to a receptor site is now routinely used to identify ligands. Optimal docking procedures need to be fast, generate reliable ligand geometries, rank the ligand conformation correctly (scoring), and thereby, estimate the binding energy. A number of studies have shown that docking algorithms are capable of finding ligands and binding conformations at a receptor site close to experimentally determined structures. These algorithms are equally applicable to the identification of multiple proteins to which a small molecule can bind. The application of this approach may facilitate the prediction of either unknown and secondary therapeutic target proteins or side effects and toxicity of particular drugs. In computational structure-based drug design, the evaluations of scoring functions are the cornerstones to the success of design and discovery. Many approaches have been explored to improve their reliability and accuracy, leading to three families of scoring functions. These are force-field-based, knowledge-based and empirical-based.[20]

SCORING FUNCTION

Scoring functions are normally parameterized (or trained) against a data set consisting of experimentally determined binding affinities between molecular species similar to the species that one wishes to predict.

Types:

1. Force field based - Force field affinities are estimated by summing the strength of intermolecular van der Waals and electrostatic interactions between all atoms of the two molecules in the complex.

2. Empirical - based on counting the number of various types of interactions between the two binding partners. Counting may be based on the number of ligand and receptor atoms in contact with each other or by calculating the change in solvent accessible surface area complex compared to the uncomplexed ligand and protein. These interactions terms of the function may include for example: hydrophobic-hydrophobic contacts, hydrophobic-hydrophilic contacts, number of hydrogen bonds, number of rotatable bonds immobilized in complex formation.

3. Knowledge-based (also known as statistical potentials) - based on statistical observations of intermolecular close contacts in large 3D databases which are used to derive "potentials of mean force". This method is founded on the assumption that close intermolecular interactions between certain types of atoms or functional groups that occur more frequently than one would expect by a random distribution are likely to be energetically favorable and therefore contribute favorably to binding affinity.[21]

Absorption, Distribution, Metabolism and Excretion (ADME) analysis

For a drug to be pharmacologically active and exert the action it should possess pharmacokinetic properties like Absorption, Distribution, Metabolism and Excretion. In the field of drug research and development many drug failures do occur, such that the drug may fail to undergo those properties satisfactorily.

This has to be ruled out earlier in the process of drug discovery. Many in-vitro studies are more frequently used to evaluate ADME properties. Some computational methods (in silico tools) have been evolved to investigate the most suitable drug molecules.

In silico modeling serves for two main functions in predicting those (ADME) properties i.e,

- Earlier investigation of designing compounds and compound libraries in order to reduce the risks at later.
- Optimizes the screening and testing, most probably by focusing only the more active compounds.
- A deep rooted knowledge in understanding the relationship of ADME parameters and the underlying (drug likeness property) molecular structural features to which it depends on.
- It enhances in elaborating this session of interest to the area of posology where it gives information about the drug dosage and frequency. This in turn reflects issues on bioavailability, crossing various biological membranes like brain, ocular and dermal penetration.

These are the essential factors and criteria to look in, for a drug to be pharmacologically active and execute as the most successful clinical candidate in the pharmaceutical research.

Prediction of ADME related parameters

Absorption:

To investigate this property in silico model used simple parameters like log D (diffusion coefficient) and polar surface area are the descriptors for hydrogen bonding capacity and (partition coefficient) log P values should fall under the prescribed values as per the rule of thumb which determines the absorption.

Bioavailabilty:

Factors like size and shape of the molecule, lipophilicity and flexibility determines the bioavailability.

Blood Brain Barrier Penetration:

In order for a drug to cross the blood brain barrier (molecule targeted to brain).Rule of thumb says that log P values should be closer to 2 with a molecular mass of <450 Da and or with a polar surface area (PSA) < 100 A are likely to possess.

Dermal and Ocular Penetration:

For dermal and ocular route it should satisfy the existing parameters like (Partition coefficient) log P for aqueous solubility, molecular weight and molecular flexibility.

Metabolism:

Various in silico approaches exist in evaluating the metabolism namely QSAR and 3D QSAR etc apart from those computational chemists have updated the structural details in the data bases and tools for predicting metabolism. Simultaneously it reveals the metabolic information as well as the toxicity related to the molecular fragments by which the drug molecule undergoes the metabolism.[60]

Evaluation of in silico toxicity:

Toxicity is one of the major criteria to be considered for a molecule to shine as a successful clinical candidate in the pharmaceutical research. About ~ 20 -40% of the drug failures fall under this category. Commercial in silico tools estimates toxicity and provides information by the use of QSAR (parameters and descriptors), scientific literatures and to some extent in abstracting issues from humans.

In silico approaches like OSIRIS property explorer predicts carcinogenicity, mutagenicity, teratogenicity, immune toxicology, irritation, sensitization etc. In addition newly updated in silico tools helps in evaluating hepato, neuro and cardiotoxicity.

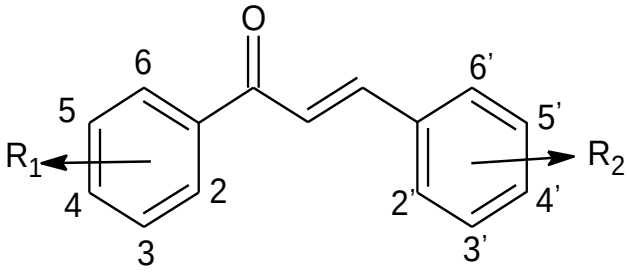
CHALCONE

Chalcone is the trivial name given to the α,β-unsaturated ketones obtained by condensing an aromatic aldehyde with an aryl methyl ketone in the presence of a base. They are designated structurally as Ar CH=CHC (O) Ar´ and their IUPAC name is 1, 3-Diphenyl-2-propene-1-one. The term "chalcone" was first used by Kostanecki.[22]

Chalcones are abundantly present in nature, from ferns to higher plants. During 1960"s and 70"s many chalcones have been reported to be isolated from the various parts of plants: buds, leaves, blossoms, heart wood, roots, seeds, flowers, and inflorescence. These compounds exist both in free and combined states either in the form of chalcones or glycosides respectively. These compounds have been found to carry many different substituents like methyl, isopentyl, methoxy and hydroxyl, which may be present either on ring A and/or ring B of the chalcone molecule.

Chalcones widely distributed in dilatory foods such as spices, tea, beer, fruits and vegetables and have been recently a subject of great interest for their pharmacological activities. According to Francisco A. et al. the major dietary source of dihydrochalcones is apples. The US FDA (Food and Drug Administration) and EU (European Union) have approved the neohesperidin dihydrochalcone to be used as sweetener in various foods like non-alcoholic soft drinks, desserts and confectionery etc. at concentrations in the range 10-400 mg kg-1 (or mg l-1) or as a flavor modifier at concentrations of up to 5 mg kg-1.[23]

Chemistry of chalcones



In this structure, the group ─CH=CHC (=O) is known as the chalcone functionality or chalcone moiety or ketoethylenic group. Due to this functionality, chalcones are also called α, β-unsaturated carbonyl systems or α,β-unsaturated ketones. The parent member of the chalcone series is benzylideneacetophenone. Other names given to chalcone are phenyl styryl ketone, β-phenylacrylophenone, γ-oxo-α, γ- diphenyl-α-propylene, and α-phenyl-β-benzoylethylene.

Chalcones have a diverse array of groups on the two aromatic rings of 1, 3- Diaryl-2-propene-1-one, as shown in the above structure, where the substituents R1 and R2 may be same or different and they may be present anywhere on the two rings. Moreover, R1 or R2 may not necessarily be a single substituent i.e. more than one substituent may be present on any of the two rings. Also, the two aromatic rings may be homocyclic or heterocyclic.[24]

Claisen Schmidt Condensation Reaction

The reaction between a ketone and a carbonyl compound lacking an alpha-Hydrogen (Cross Aldol condensation) is called Claisen-Schmidt condensation. These reactions are named after two of its pioneering investigators Rainer Ludwig Claisen and J. G. Schmidt, who independ ently published on this topic in 1880 and 1881.[25]

Mechanism

The first part of this reaction is an aldol reaction, the second part a dehydration,an elimination reaction (involves removal of a water molecule or an alcohol molecule). Dehydration may be accompanied by decarboxylation when an activated carboxyl group is present. The aldol

addition product can be dehydrated via two mechanisms; a strong base like potassium *t*-butoxide, potassium hydroxide or sodium hydride in an enolate mechanism or in an acid-catalyzed enol mechanism.[26]

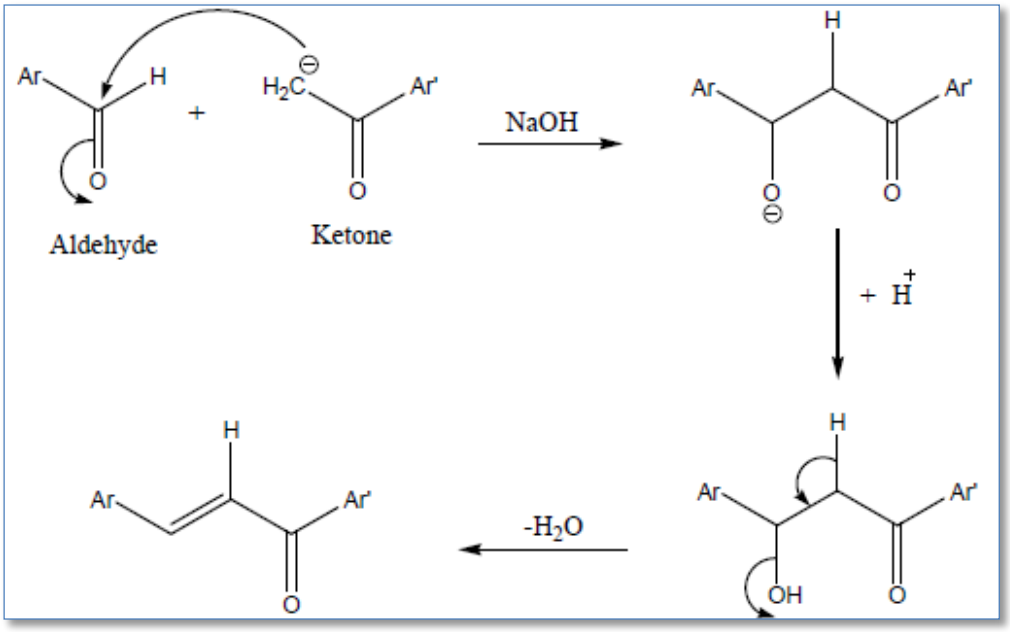


Fig 5: Mechanism of the Claisen Schmidt reaction

Pharmacological Profile of Chalcones

Chalcones, either natural or synthetic, are known to exhibit a broad spectrum of various biological activities. The presence of α, β-unsaturated carbonyl moiety as well as of substituted aromatic ring renders the chalcones biologically active[26]. Some substituted chalcones and their derivatives, including some of their heterocyclic analogues have been reported to possess strong biological properties which have been proved detrimental to the growth of microbes, tubercle bacilli, malarial parasites and intestinal worms[27]. Many chalcones have been claimed to be toxic to various animals and insects and have also shown inhibitory effects on several enzymes and herbaceous plants.[28] A few major biological activities which have been reported to be associated with chalcones include: anti-inflammatory, antifungal, antiviral, antioxidant, antimalarial, antituberculosis, analgesic, anti- HIV and antitumor activities. Quinoline based chalcones have been reported to possess antimalarial activity.[29]

CHARACTERIZATION

IR SPECTROSCOPY

Infrared spectroscopy is certainly one of the most important analytical techniques. One of the great advantages of infrared spectroscopy is that virtually any sample in any state may be studied.

Infrared spectroscopy is a technique based on the vibrations of the atoms of a molecule. An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule.[30]

Infrared spectroscopy is used to obtain information on the molecular structure of the sample. The infrared spectrum is related to the vibrations of molecules and is unique for each compound, like a fingerprint for a person.

The possible characteristic bands obtained in IR spectroscopy are:

- 3300 - 3540 cm^{-1} N-H Stretching vibration
- 3650 - 3200 cm^{-1} O-H stretching vibration
- 1690 - 1630 cm^{-1} C=N stretching vibration
- 2960 - 2850 cm^{-1} C-H aliphatic stretching vibration
- 3100 - 3000 cm^{-1} C-H aromatic stretching vibration.[31]

NMR SPECTROSCOPY

Nuclear magnetic resonance spectroscopy is one of the most important characterization method. The NMR phenomenon is based on the fact that nuclei of atoms have magnetic properties that can be utilized to yield chemical information.[32]

The NMR spectrum proves to be of great utility in structure elucidation because the properties it displays can be related to the molecular structure. The *chemical shift* of a particular nucleus can be correlated with its chemical environment, the *scalar coupling* (or *J-coupling*) indicates an indirect interaction between individual nuclei, mediated by electrons in a chemical bond, and, under suitable conditions, the area of a resonance is related to the number of nuclei giving rise to it.[33]

The possible characteristic peaks obtained are:

- Aromatic and Heteroaromatic proton 6 – 8 δ
- Phenolic proton 4 – 12 δ
- Aliphatic proton 1.5 – 4.5 δ

MASS SPECTROSCOPY

Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds, and to illuminate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities[34].

Atoms or molecules are passed into a beam of high-speed electrons. The high-speed electrons knock electrons off the atoms or molecules being analyzed and change them to positive ions. The applied electric field then accelerates these ions through a magnetic field, which deflects the paths of the ions. The most massive ions are deflected the smallest amount, which causes the ions to separate. The gas phase ions are sorted in the mass analyzer according to their mass-to-charge (m/z) ratios and then collected by a detector. In the detector the ion flux is converted to a proportional electrical current. The data system records the magnitude of these electrical signals as a function of m/z and converts this information into a mass spectrum. It is used to identify, quantify, and elucidate structures of chemical compounds.[35]

ANTITUBERCULAR ACTIVITY

From the earlier to the present status, tuberculosis is existing with (extreme & multi-drug resistance), increased susceptibility in transmission, their complex pathophysiology leading to higher risks of infections to other organs and increases the rate of mortality. So essentially there is an urgent and rapid need of drugs to combat this disease, which in turn it is screened for anti tuberculosis activity.

The following methods are used for the invitro evaluation of antitubercular activity.

- Resazurin Micro Plate Assay(REMA)
- Nitrate Reductase Assay(NRA)
- Micro Plate Alamar Blue Assay(MABA)
- Middle Brook 7H11 Agar Dilution Assay
- BACTEC System
- Luciferase Reporter Phage Assay.[36]

The synthesized compounds can be evaluated for anti tubercular activity by any of the above methods.

MICROPLATE ALAMAR BLUE ASSAY

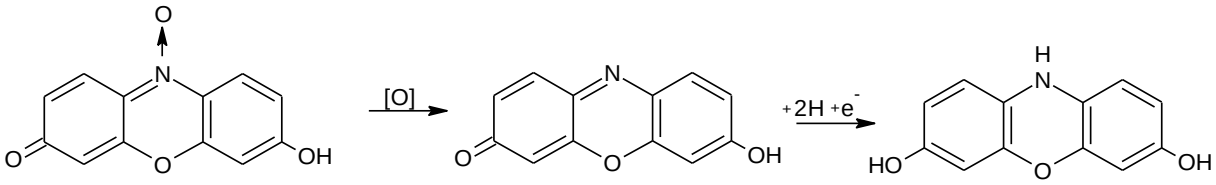
The Microplate alamar blue assay (MABA) method was used to evaluate antitubercular activity of synthesized compounds against mycobacterial strain *Mycobacterium tuberculosis* H37Rv. The medium used for this evaluation is 7H9GC media.

Principle

Resazurin is purple in color. Resazurin reduces in two steps, irreversibly to resorufin and then reversibly to dihydroresorufin, providing color changes from purple to pink to colorless. The tubercle bacteria reduce resazurin which causes disappearance in purple color. The synthetic

compounds cause reoxidation which is pink in colour. The color intensity is measured in fluorimeter.[37] The percentage inhibition is defined as

$$\% \text{ Inhibition} = \frac{\text{Test well fluorescence}}{\text{Mean fluorescence of triplicate wells}}$$



Resazurin Resorufin

Method

Antitubercular activity was evaluated against *Mycobacterium tuberculosis* H37Rv using Micro plate alamar blue assay (MABA) method. Antitubercular susceptibility test was performed in black, clear bottomed, 96-well micro plates in order to minimize background fluorescence. Initial drug dilutions were prepared in dimethylsulfoxide and subsequent two fold dilutions were performed in 0.1 ml of 7H9GC media in the micro plates. 100ml of 2000 CFU/ ml of *M. tuberculosis* H37Rv were added to each well of 96 well microtitre plate containing test compounds. Three control well plates containing drug and medium, bacteria and medium and medium only were also prepared. All microtitre plates were incubated at 37°C for seven days. At day 7 of incubation Alamar blue dye solution (20 µl Alamar blue solution and12.5 ml of 20% Tween 80) was added to all the wells and plates were re-incubated at 37°C for 24 h. Fluorescence was measured in a fluorimeter and MIC was determined.[38]

Advantages

- It has accurate time-course measurement
- It has high sensitivity and linearity
- It involves no cell lysis
- It is ideal for use with post measurement functional assays

- It is flexible as it can be used with different cell models
- It is scalable and can be used with fluorescence and/or absorbance-based instrumentation platforms
- Finally, it is non-toxic, non-radioactive and is safe for the user.

Aim and objectives

Aim and objective

AIM

The aim of this project is to develop potential antimycobacterial agents.

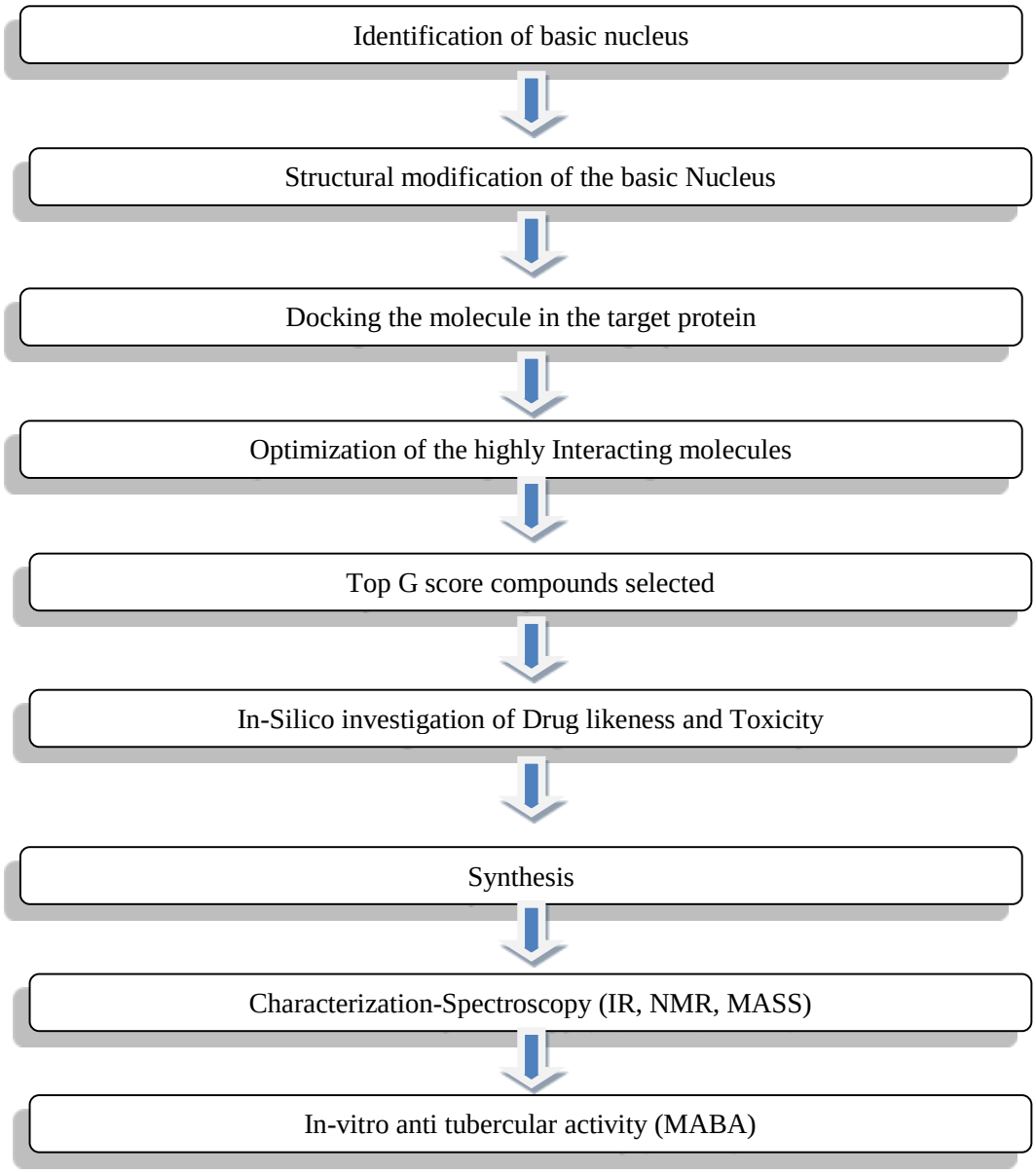
OBJECTIVES

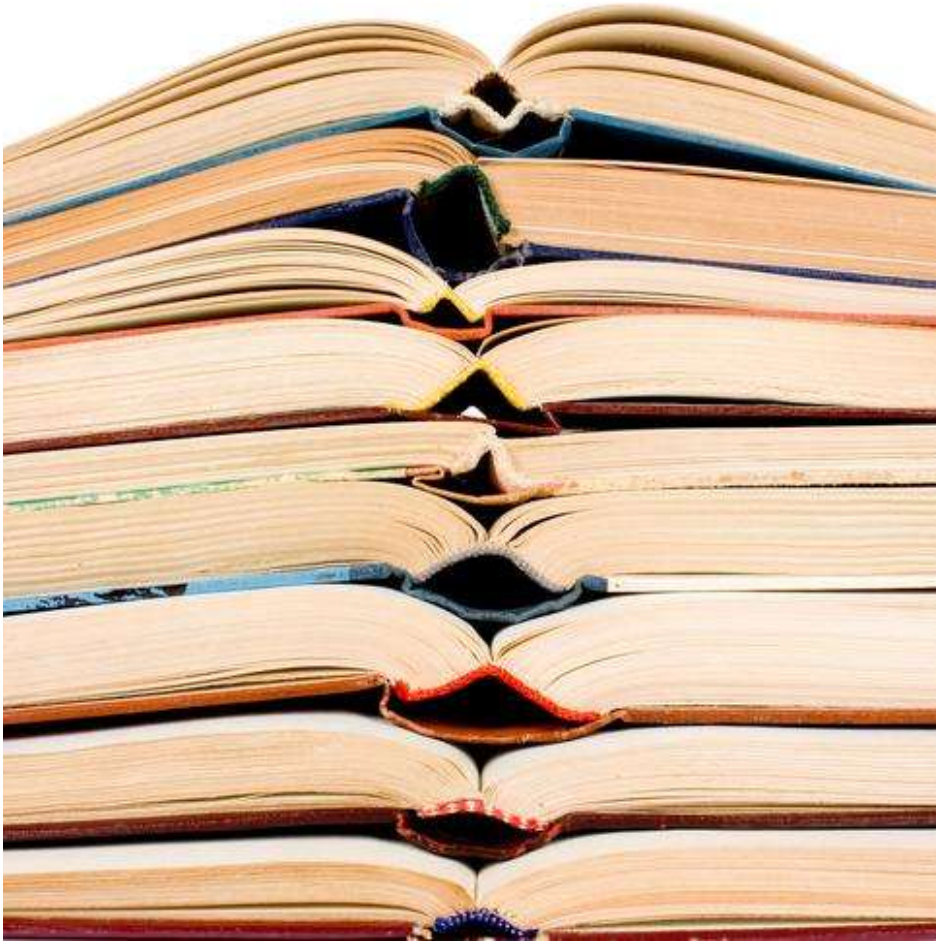
The objective of the project is to design and synthesize some compounds which will act on L, D Transpeptidase 2 and inhibit the cell wall synthesis of *M.tuberculosis.*

THE PLAN OF WORK

- Design of L, D Transpeptidase 2 inhibitors by docking studies
- Insilico Prediction of Drug Likeness and Toxicity
- Laboratory synthesis of the top G score compounds
- Characterization of the synthesized compounds by
    - ✓ Infrared Spectroscopy
    - ✓ Nuclear Magnetic Resonance Spectroscopy
    - ✓ Mass Spectroscopy
- In-vitro anti tubercular activity of synthesized compounds.

The whole study was carried out according to this flow chart.



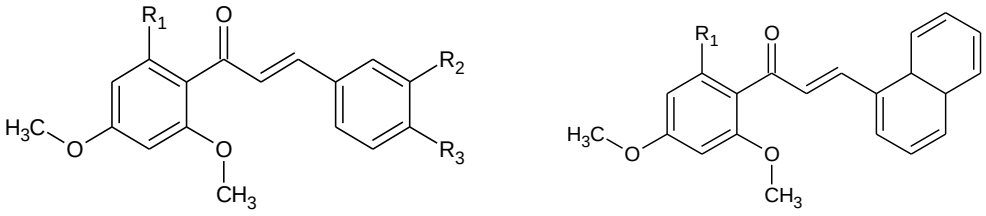


Review of Literature

LITERATURE REVIEW

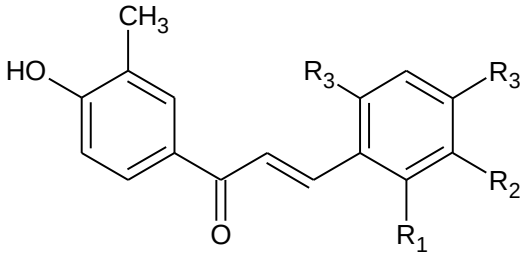
A) Reviews related to the antimycobacterial activity of chalcones

Louise Domeneghini Chiaradia et al[46]., synthesized 38 new chalcones for their anti-tubercular activity by targetting mycobacterium enzyme phosphotyrosine phosphatese. Out of the 38, 5 compounds shows good active against Mycobacterium tuberculosis PtpA at the concentration range of 5–60µg/ml.



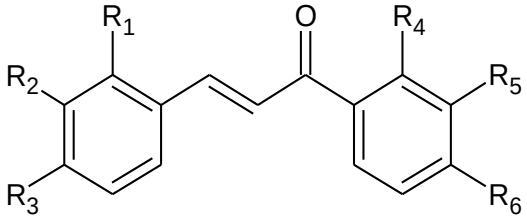
Antitubercular activity

Shahar Yar et al[47]., synthesized a novel series of eleven chalcones, which were tested for antimycobacterial activity against M. tuberculosis H37RV using a BACTEC- 460 radiometric system. Among the eleven chalcones, only six were found to be active. The compounds exhibited largest efficacy and displayed 90% inhibition at MIC ≈ 6.25 µg/ml.



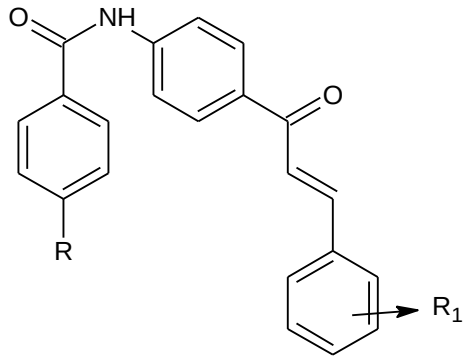
Antitubercular activity

P M Sivakumar et al[48]., synthesized and screened a series of chalcones for their anti tubercular activity. Twenty three chalcones were synthesized and were evaluated for antimycobacterial activity. Ortho chloro substitutions at A and B-ring was found to favour activity. Methylsulfonyl chalcones exhibit very good activity.



Antitubercular activity

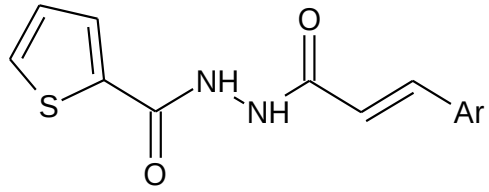
Jahirul Islam Talukdar and Monica Kachroo[49]., synthesized chalcones and tested for its Antitubercular and antibacterial activity. It was found that all the compound possessed antibacterial activity, on the other hand, the compound N-{4-[3-(2,4-dimethoxy-phenyl)-acryloyl]-phenyl}-4- methoxy benzamide exhibited antitubercular activity at concentration of 25 µg/ml whereas all other derivatives of the series exhibited activity at a concentration of above 50 µg/ml.



Antibacterial and Anti tubercular activity

Dr. Mrunmayee Toraskar et al[49]., synthesized thiophene derivatives of chalcones. The compounds were evaluated for in vitro anti-fungal, anti-tubercular and anti-oxidant activity. All the synthesized compounds showed very good in vitro anti-fungal activity against C. albicans by using fluconazole as standard. Compounds with electron donating groups on the aldehydic

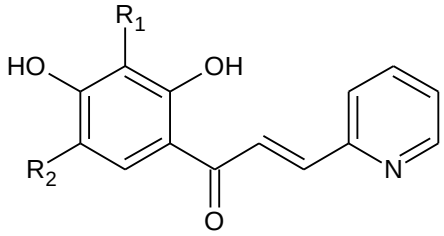
phenyl ring showed very good anti-tubercular activity against mycobacterium tuberculosis. Compounds with substituted 4-methoxy group at phenyl ring showed good anti- oxidant activity may be due to its electron donating ability.



Antifungal, Antimycobacterial and Antioxidant activity

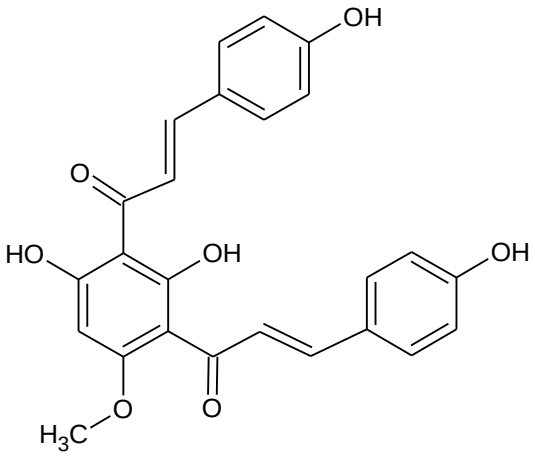
B) **Reviews related to other biological activities of chalcones**

Seema I. Habib et al[51]., synthesized and screened a series of chalcones for their antimicrobial activity. The screening of antimicrobial data revealed that compounds with pyridine nucleus showed much activity than pyrrole.



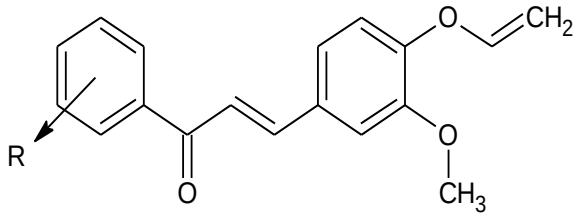
Antimicrobial activity

Susanne *et al.*[56], synthesized the 3′- coumaroyl-2′, 4, 4′-trihydroxy-6′-methoxychalcone, were structurally derived from helichrysetin. This compound showed the highest cytotoxic activity against HeLa cells with an IC50 value of 7.3 ± 0.4 μM. Anti-oxidative effects were determined in the ORAC assay and revealed very strong activity for the compound given below.



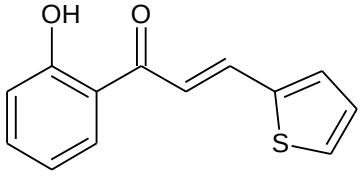
Anti-oxidant activity

Bathelemy Ngamenil et al[52]., synthesised and evaluated anticancer activity for O-allylchalcone derivatives. The series of new O-allylchalcone derivatives prepared by a Claisen-Schmidt condensation reaction have good ability to kill tumor cells in vitro.



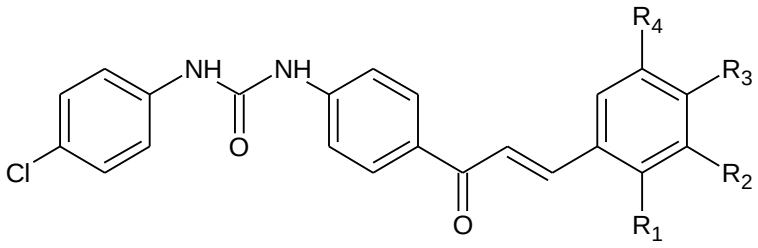
Anticancer activity

Won et al[53]., synthesized chalcones and screened for their anti-inflammatory activity. This synthesised chalcones has the ability to inhibit chemical mediators released from mast cells, neutrophils, macrophages.



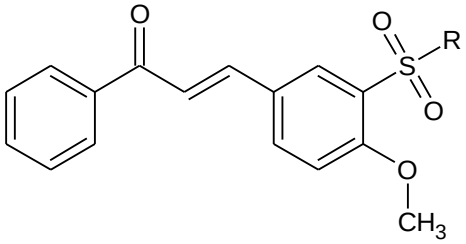
Anti-inflammatory activity

Domínguez et al[54]., reported phenylurenyl chalcone derivatives, with substitution in ring B (IC50 = 1.76-10 μM) as potent growth inhibitors against in vitro cultured P.falciparum.



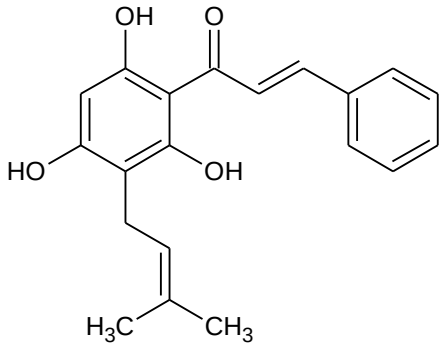
Antimalarial activity

Carla *et al*[55]., showed a new set of sulfonamide 4- methoxychalcone derivatives were synthesized and which shown antileishmanial activity against Leishmania braziliensis promastigotes and intracellular amastigotes and determined its cell toxicity profile. Interestingly all compounds presented a concentration dependent antileishmanial profile and the benzylamino derivative showed a biological activity better than pentamidine.



Antileishmanial activity

Dong *et al*[56]., studied the series of prenylated flavonoids. According to the estimated result, eleven molecules were selected and synthesized. Their vasodilatory activities were determined experimentally in rat aorta rings that were pretreated with phenylephrine (PE). Structure–activity relationship (SAR) analysis revealed that flavanone derivatives showed the most potent activities, while flavone and chalcone derivatives exhibited medium activities.



Vasodilatory activity

C) Review related to the target

Sabri B. Erdemli et al[57] studied that the traditional β-lactam antibiotics that disrupt the D,D-transpeptidases are not effective against mycobacteria as mycobacteria rely mostly on β-lactam insensitive L,D-transpeptidases for biosynthesis and maintenance of their peptidoglycan layer. This plays a major role in drug-resistance and persistence of infections. The crystal structure at 1.7 Å resolution of the Mtb L,D-transpeptidase LdtMt2 contains a bound peptidoglycan fragment which provides information about catalytic site organization as well as substrate recognition by the enzyme. Based on the structural, kinetic, and calorimetric data, a catalytic mechanism for LdtMt2 has been proposed.

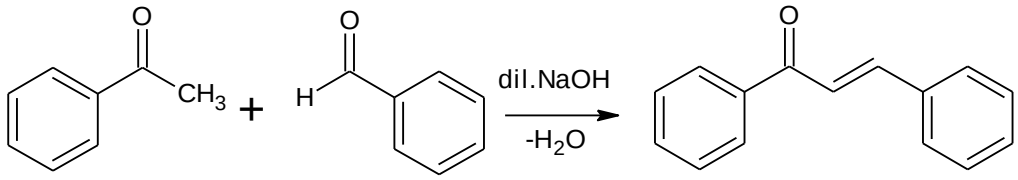
Soumya De and Lawrence P. McIntosh[58] investigated the structural and dynamic basis for the unexpected inhibition of peptidoglycan crosslinking L, D transpeptidases by carbapenam antibiotics.

Dominic Both et al [59] studied the crystal structure of the protein and summarized that the transpeptidase Ldt_{Mt2} catalyzes the formation of the cross-links characteristic of the peptidoglycan layer in the Mycobacterium tuberculosis cell wall. Bioinformatics analysis suggests that the extramembrane part of the enzyme consists of three domains: two smaller domains (denoted as A and B domains) and a transpeptidase domain (the C domain) at the C-terminus. The crystal structures of two fragments comprising the AB domains and the BC

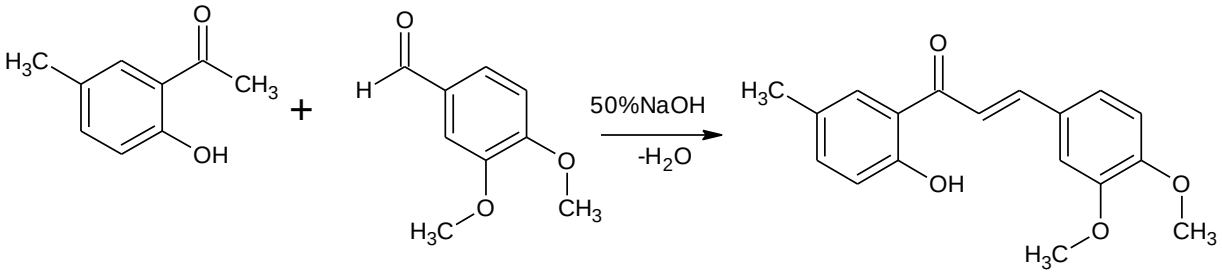
domains have been determined. Combining the structures of the two fragments provides a view of the complete three-domain extramembrane part of Ldt_{Mt2} and shows that the protein extends at least 80– 100 A ° from the plasma membrane into the peptidoglycan layer and thus defines the maximal distance at which crosslinks are formed by this enzyme.

D) Reviews related to synthesis of Chalcones

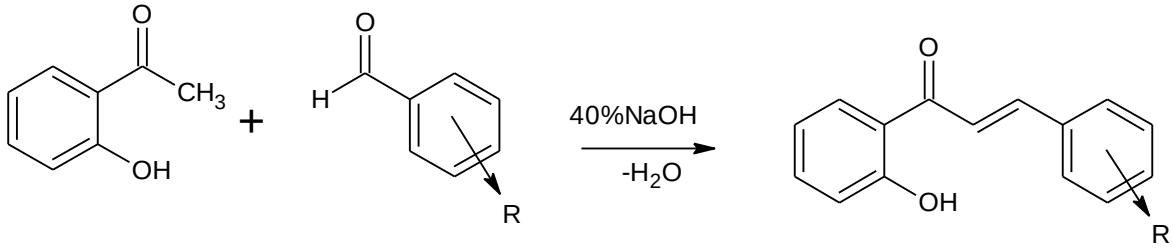
Claisen Schmidt condensation[42]



Arshi Naqvi et al[43]., synthesised chalcones by stirring method using 50% NaOH.



Nitin Kumar et al[44]., synthesised chalcones by using 40% NaOH.



E) Reviews related to the evaluation of anti tubercular activity by MABA

1) **Scott G.Franzblau et al**[36], studied MIC determination by MABA. A colorimetric, microplate- based Alamar blur Assay (MABA) method was used to determine the MICs of Isoniazid, rifampin, streptomycin and ethambutol for 34 Peruvian Mycobacterium tuberculosis isolates and the H37Rv strain by using bacterial suspensions prepared directly from media. The MABA is a simple, rapid, low-cost, appropriate technology which does not require expensive instrumentation and which makes use of a nontoxic, temperature-stable reagent

2) **Sephra N.Rampresad et al**[37] studied the various applications of Alamar blue as an indicator. Alamar Blue is an important redox indicator that is used to evaluate metabolic function and cellular health. The Alamar blue bioassay is being utilized to access cell viability and cytotoxicity in a range of biological and environmental system and in a number of cell types including bacteria, yeast, fungi,protozoa.

3) **Jose de Jesus Alba-Romero et al**[38] applied the Alamar Blue assay to determine the susceptibility to anti-tuberculosis pharmaceuticals. The results showed that the MABA test is fast and easy to apply. It is a very reliable method of determining the drug susceptibility to pharmaceuticals.

Materials and Methods

MATERIALS AND METHODS

1. DRUG DESIGN

In this research Glide® (grid based ligand docking with energetics) program was used for screening a large number of compounds. Glide automatically searches for favorable interactions between ligand molecule and the receptor in different conformations. Docking procedure using glide program includes the following steps,

1. Protein preparation
2. Receptor grid generation
3. Ligand preparation
4. Ligand docking
5. Visualizing docking poses

Protein preparation

Protein data bank (PDB) file, which is the crystallized structure of the receptor/protein is imported from Protein Data Bank with the following PDB id: 3VAE(L,D Transpeptidase 2), resolution 2.8Å, preprocessed involving addition of hydrogen, assigning bond order, finding overlaps, creating zero order bond to metals, creating disulfide bonds, Filling missing side chains and loops using prime option. The water molecules, co-factors and unwanted chains were deleted. And the energy minimization has been done so that it will be ready for grid generation. The PDB file was selected based on its species, X-ray crystallography or NMR spectroscopy, resolution value, external ligand and presence co-factos.[39]

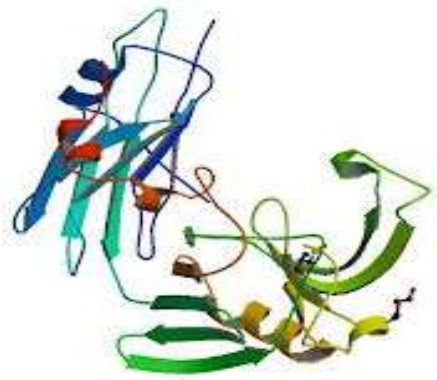


Fig 6: 3VAE

Receptor Grid Generation

Once the protein has been prepared, the grid has to be generated which is the critical process, It includes defining the active site in the protein (receptor). The prepared protein file was loaded into the workspace the active site residues were found and picked and the length for docking the ligand to the protein is given as 10Å. The grid was generated on giving start in the grid generation tab. The grid output file obtained as zip file format was utilized for further docking process.

Ligand Preparation

Ligand preparation process consists of a series of steps that include conversions, applying corrections to the structures drawn, generating variations on the structure, eliminating unwanted structures and optimizing the structures.

Variations on the structure can be made by addition of hydrogen atoms, removal of unwanted molecules, neutralizing charged groups. The structure can be optimized by generating ionization states, generating tautomers, filtering the structure on the basis of Lipinski's rule of five.

Ligand docking

After the generation of the grid, the prepared ligands were docked to see the interaction with the active site of the protein. There were hydrophobic, hydrophilic and Vanderwaal's interaction. The strength of the interaction was different for different ligand molecules. During the docking procedure, conformation of the ligand was retained and extra precision (xp) mode was selected. In this procedure the following constraints like active site and rotatable groups have been checked. Around 1000 molecules were docked and these with best scores were taken for synthesis and screening purpose.[41]

Visualization of the docking poses

Once the molecules were docked, then they were visualized for interactions, score and some other parameters like log P value and ionization value. There were interactions like hydrogen bonding, hydrophobic interaction, Vanderwaal's interaction between the receptor and the ligand. Based on the interaction and score obtained, the molecules were categorized into hit and flop. The hit molecules were accounted for synthesis and screening.

2. IN-SILICO SCREENING OF DRUG LIKENESS

For a drug to be pharmacologically active and exert action it should posses pharmacokinetic properties like absorption, distribution, metabolism and excretion. Many drug failures occur due to unfavourable ADME properties in the field of drug research and development. This has to be ruled out earlier in the process of drug discovery. Some computational methods (in silico tools) have been evolved to investigate the most suitable drug molecules before synthesis.

Lipinski's rule of five also known as the **Pfizer's rule of five** is a rule to evaluate drug likeness. It is used to predict whether a molecule is likely to be orally bio-available or to evaluate drug likeness.[61]

Lipinski's rule

This rule is used to predict whether a molecule is likely to be orally bio-available or to evaluate drug likeness. The rule was formulated by Christopher A. Lipinski in 1997.The rule states that for drug likeness the molecule should have the following properties:

- Molecular weight less than 500 Daltons
- Calculated log P value less than 5
- Less than 10 hydrogen bond acceptor groups(eg.-O-, -N-,etc)
- Less than 5 hydrogen bond donor groups (eg. NH, OH, etc)
- Less than 10 rotatable bonds

The designed and docked molecules are screened in silico using **Molinspiration Cheminformatics Software** to evaluate drug likeness. This tool is quick and easy to use. It can be accessed online for calculation of important molecular properties such as logP, polar surface area, number of hydrogen bond donors and acceptors as well as prediction of bioactivity score for the most important drug targets like GPCR ligands, kinase inhibitors, ion channel modulators, nuclear receptors.[62]

3. IN SILICO TOXCITY PREDICTION

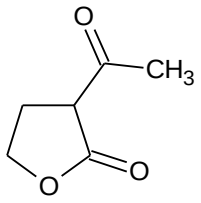
Toxicity screening is done insilico using OSIRIS Property Explorer. It is a web based software available on the Organic Chemistry Portal[63]. Using this prediction tool, mutagenicity, tumorogenicity, skin irritancy, and reproductive effects can be calculated. The prediction properties depends on a precompiled set of structure fragment that gives rises to toxicity alerts, if they are found in the structure currently drawn. These fragment lists is created by rigorously shredding all compounds in the data base known to be active in a certain toxicity class. During the shredding any molecule is first cut at every rotatable bonds leading to a set of core fragments.[64] Osiris software is used to calculate various drug relevant properties of chemical structures. The results are colour coded. The green colour represents that the compound is non-toxic. Yellow and red colour indicates moderate and severe toxicity of the chemicals respectively.

4. SYNTHETIC METHODOLOGY

REACTANT PROFILE

1. ALPHA ACETYL BUTYROLACTONE

Structure:



Synonym: 3-acetyldihydrofuran-2(3*H*)-one

Molecular Formula: $C_6H_8O_3$

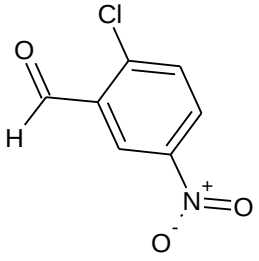
Molecular Weight: 128.12

Description: Colourless to light yellow liquid

Boiling Point: 107°C

2. 2-CHLORO 5-NITRO BENZALDEHYDE

Structure:



Synonym: 2- chloro-5-nitro benzaldehyde

Molecular Formula: $C_7H_4ClNO_3$

Molecular Weight: 185.56

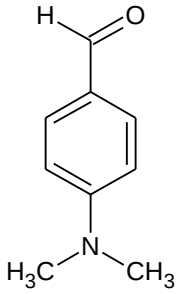
Solubility: Soluble in DMSO,chloroform, acetonitrile.

Description: Light yellow to beige yellow crystalline powder

Melting Point: 76°C

3. Para- DIMETHYL AMINO BENZALDEHYDE

Structure:



Synonym: 4- (dimethylamino) benzaldehyde

Molecular Formula: $C_9H_{11}NO$

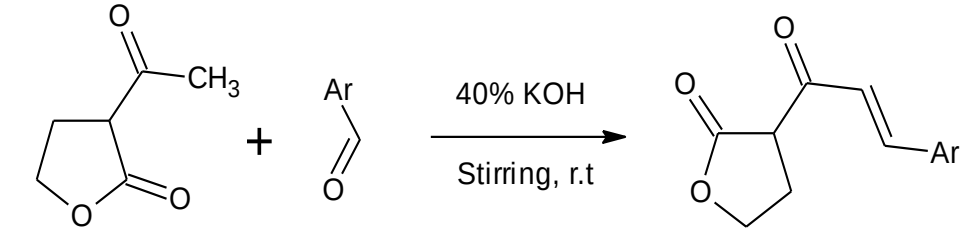
Molecular Weight: 149.18

Solubility: Soluble in chloroform,acetonitrile,hot ethanol.Slightly soluble in water.

Description: White crystalline powder

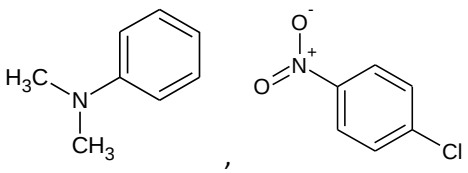
Melting Point: 73°C

SYNTHESIS[42-44]



alpha acetyl butyrolactone aldehyde Chalcone

Ar-



PROCEDURE

A mixture of alpha acetyl butyrolactone (0.01 moles) and aryl aldehyde (0.01 moles) was stirred in ethanol (15 ml) and then an aqueous solution of potassium hydroxide (40%, 15 ml) was added to it. The reaction mixture was stirred at room temperature for 6 hours. The completion of the reaction was monitored by TLC. After the completion of the reaction, the reaction mixture was poured into cold water and acidified with hydrochloric acid (10%). The solid that separated was filtered and dried. It was recrystallised with ethanol.[42,43]

RECRYSTALLISATION

To the synthesized compound, warm ethanol was added and it was heated. The hot solution was filtered and the filtrate was collected in a china dish and allowed to cool. On cooling, crystals appeared.

CHARACTERIZATION

A) MELTING POINT

The melting point of the synthesized compound was determined by one end open capillary method.

B) THIN LAYER CHROMATOGRAPHY

The reactants and products were dissolved in ethanol. It was spotted on the TLC plate.

Stationary phase: Pre-coated Silica gel GF plates
Mobile phase: Benzene:Chloroform:Ethanol (5:4:1)
Detection: UV chamber

A single principle spot for the product and the absence of secondary spots and spots for parent compounds confirmed the purity of the compound.

C) IR SPECTROSCOPY

IR Spectroscopy helps to ascertain the presence and absence of the functional group. The synthesized compound was made into a pellet with potassium bromide by pressed pellet technique using pellet press (Model No: M15). The pellet was mounted on the pellet disc and percentage transmittance was recorded in ABB IR Spectrophotometer (Model No: MB 3000).

D) NMR SPECTROSCOPY

Proton NMR Spectroscopy helps us to study the number of equivalent protons and their environment thereby we can ascertain the structure of the molecule. The NMR spectra were recorded on 300 MHz BRUKER Advance III NMR Spectrometer. DMSO was used as solvent.

E) MASS SPECTROSCOPY

Mass Spectroscopy enables us to establish the molecular mass of the compound. The mass spectra of the synthesized compounds were recorded in Q-Tof-Mass Spectrometer (Q-Tof micro hybrid quadrupole time of flight mass spectrometer) with electrospray ionization (ESI) and in JEOL GCMATE II GC-MS.

5. IN VITRO ANTITUBERCULAR ACTIVITY

Anti-TB activity using Alamar Blue Dye

PROCEDURE

1) The anti mycobacterial activity of compounds were assessed against M. tuberculosis using microplate Alamar Blue assay (MABA).
2) This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method.
3) Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation.
4) The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate.
5) The final drug concentrations tested were 100 to 0.2 µg/ml.
6) Plates were covered and sealed with parafilm and incubated at 37ºC for five days.
7) After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs.
8) A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.
9) The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.[45]

Results and Discussion

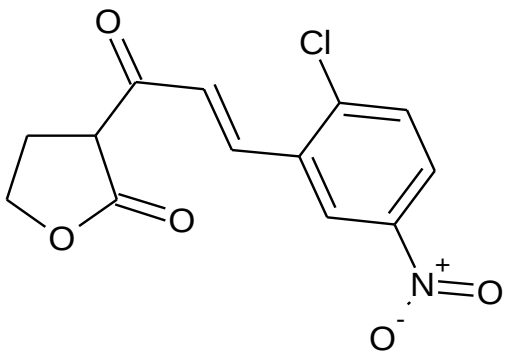
RESULTS AND DISCUSSION

1. DRUG DESIGN

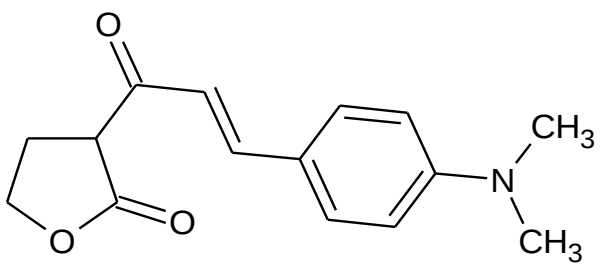
The designed molecules were docked against the target 3VAE after the active site on the protein was selected using Sitemap function. For perfect docking of the ligand into the cavity of the protein having active site, extra precision mode molecular docking was executed. During the docking procedure different poses of the ligand were generated. The ligands were docked in different poses. The best docked pose was selected based on the G.Score generated and the interactions between the protein and the ligand. Hydrogen bond interactions are seen between the compounds and the active site. Hydrophobic interactions are also seen.

DOCKING SCORE OF THE COMPOUNDS AGAINST 3 VAE

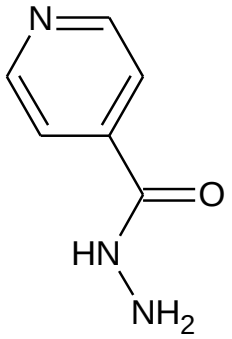
1. **SR1:** G Score: -7.04 kcal/mol



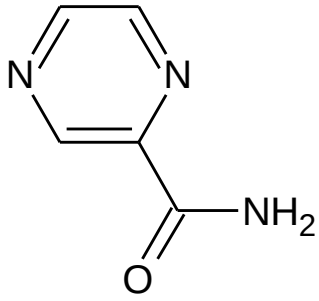
2. **SR2:** G Score: -7.64 kcal/mol



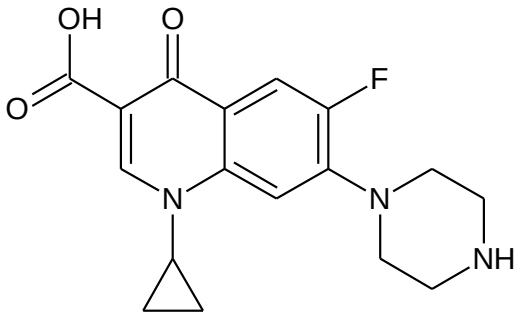
DOCKING SCORE OF THE STANDARD DRUGS AGAINST 3VAE



Isoniazid
G.Score: -5.1 kcal/mol



Pyrazinamide
G.Score: -4.3 kcal/mol



Ciprofloxacin
G.Score: -7.0 kcal/mol

COMPOUNDS DOCKED AGAINST PROTEIN 3VAE

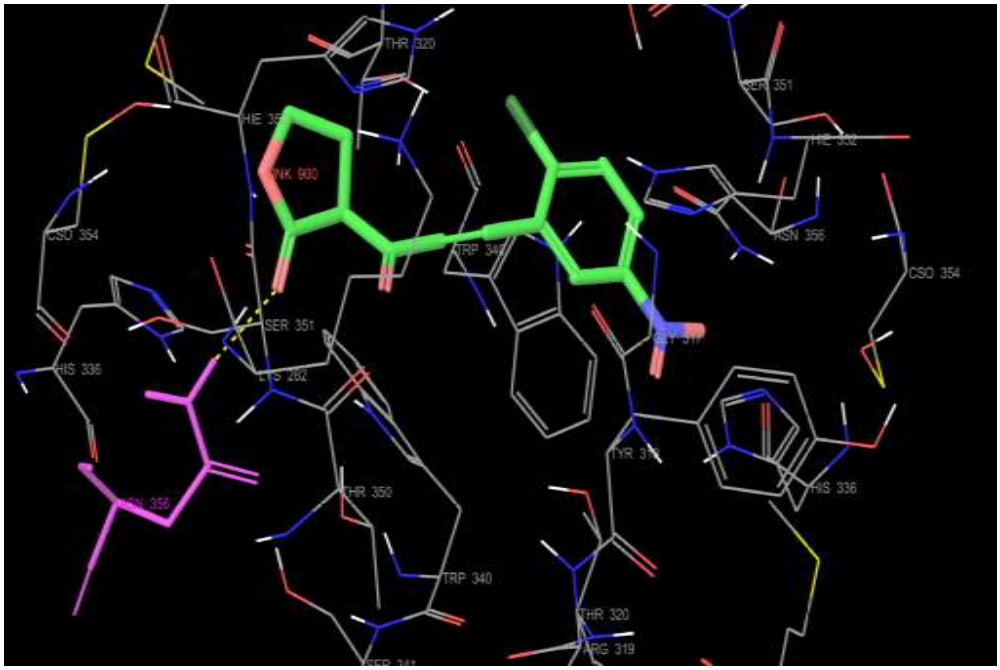


Fig 7: SR1 docked against 3VAE

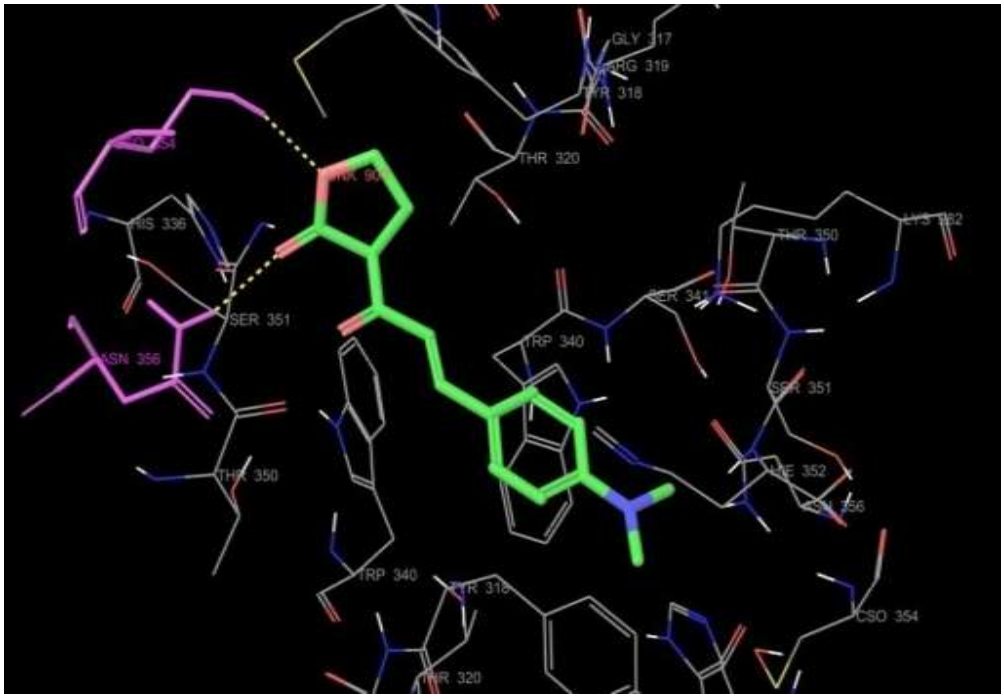
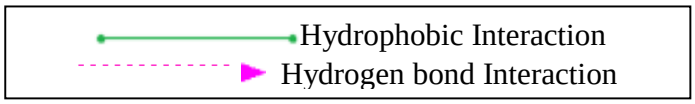


Fig 8: SR 2 docked against 3VAE

INTERACTIONS BETWEEN THE COMPOUNDS AND THE PROTEIN

Key:



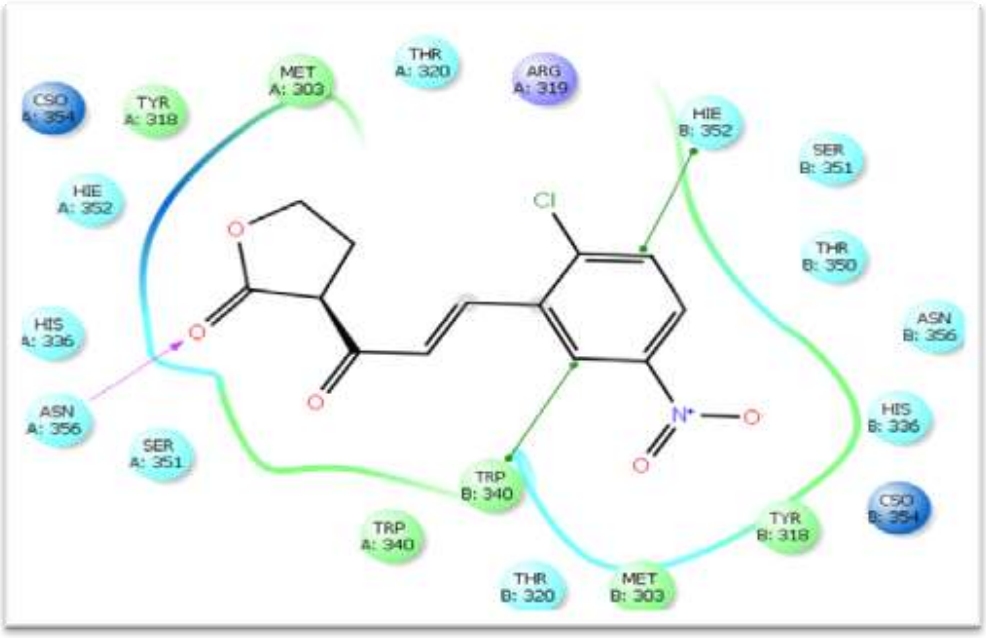


Fig 9: Interactions of SR 1 with 3VAE

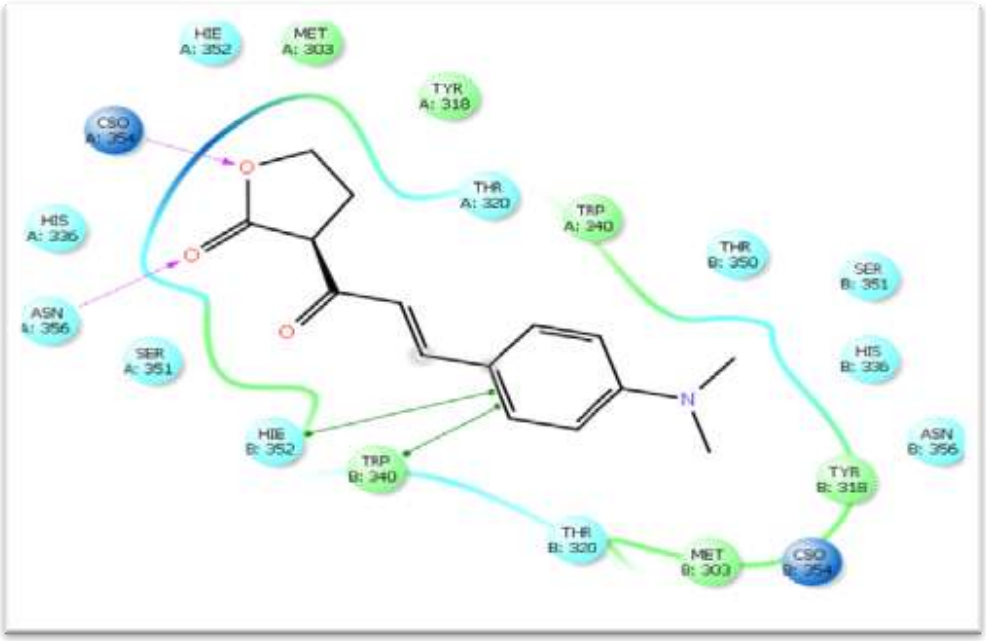


Fig 10: Interactions of SR 2 with 3VAE

Ligand	**GScore**	**DockScore**	**Lipophilic EvdW**	**PhobEn**	**Phob EnHB**	**PhobEn PairHB**	**HBond**	**Electro**	**Sitemap**	**PiCat**	**ClBr**	**LowMW**
SR1	-7.04	-7.04	-4.51	0	0	0	-1.26	-0.39	-0.56	0	0	-0.5
SR2	-7.64	-7.63	-4.51	0	0	0	-1.8	-0.44	-0.62	0	0	-0.5

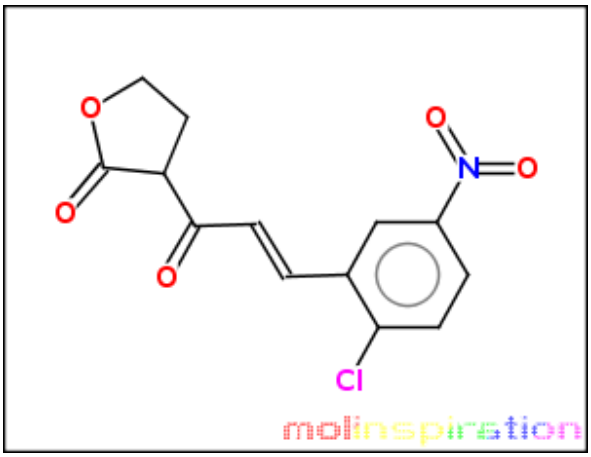
Table No 1: Rewards

Ligand	**Penalties**	**HBPenal**	**ExposPenal**	**RotPenal**	**EpikStatePenalty**	**Similarity**	**Activity**
SR1	0	0	0	0.18	0	1	-7.04
SR2	0	0	0	0.23	0	1	-7.64

Table No 2: Penalties

2. IN-SILICO SCREENING OF DRUG LIKENESS

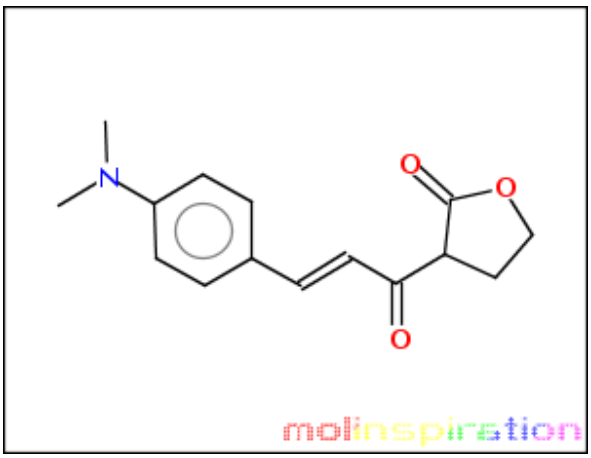
The compounds were screened for drug likeness by using Molinspiration Cheminformatics Software. On screening it was found that the ADME parameters were within the range.



Molinspiration property engine v2013.09

miLogP	1.435
TPSA	89.2
natoms	20.0
MW	295.678
nON	6
nOHNH	0
nviolations	0
nrotb	4
volume	234.871

Fig 11: Screenshot of SR 1 screening of drug likeness in Molinspiration



Molinspiration property engine v2013.09

miLogP	1.152
TPSA	46.614
natoms	19.0
MW	259.305
nON	4
nOHNH	0
nviolations	0
nrotb	4
volume	243.907

Fig 12: Screenshot of SR 2 screening of drug likeness in Molinspiration

3. IN SILICO TOXCITY PREDICTION

In silico toxicity prediction was done using OSIRIS Property Explorer. This software is available for access in the Organic Chemistry Portal. Using this prediction tool, mutagenicity, tumorogenicity, skin irritancy, and reproductive effects were calculated. The results were colour coded. The green colour represents that the compound is non-toxic. Yellow and red colour indicates moderate and severe toxicity of the chemicals respectively.

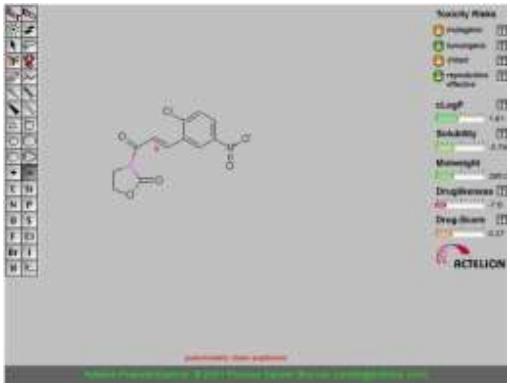


Fig 20: Toxicity prediction for SR 1

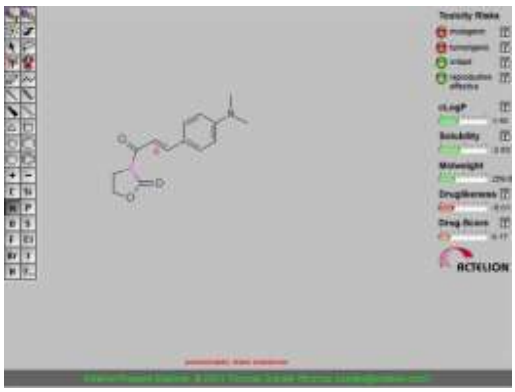


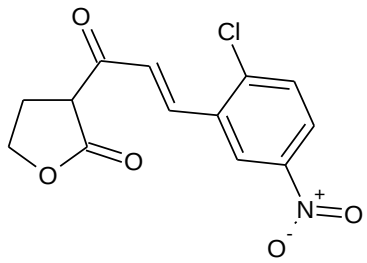
Fig 21: Toxicity prediction for SR 2

The compound SR 1 is predicted to have moderate skin irritancy and mutagenic effect where as the compound SR 2 is predicted to have severe mutagenic and tumorogenic effect.

4. SYNTHESIS AND CHARACTERIZATION

The synthetic scheme was drawn for the hit compounds from docking and the procedure for synthesis was collected from literature. The necessary chemicals of laboratory grade were procured and synthesis was carried out after the optimization of the reaction conditions. Products were obtained with a yield of about 82 % and then recrystallised. The physical properties such as appearance, solubility, melting point were recorded and uncorrected.

Analytical Data: Compound SR1



3-[(2*E*)-3-(2-chloro-5-nitrophenyl)prop-2-enoyl]dihydrofuran-2(3*H*)-one

S. No	Properties	
1.	Description	Yellowish white crystals
2.	Molecular Formula	$C_{13}H_{10}ClNO_5$
3.	Molecular Weight	295.67
4.	Melting Point	134 ºC
5.	Solubility	Partially soluble in ethanol and methanol, fully soluble in DMSO, insoluble in water

Table No 3: Physical properties of SR 1

IR SPECTROSCOPY

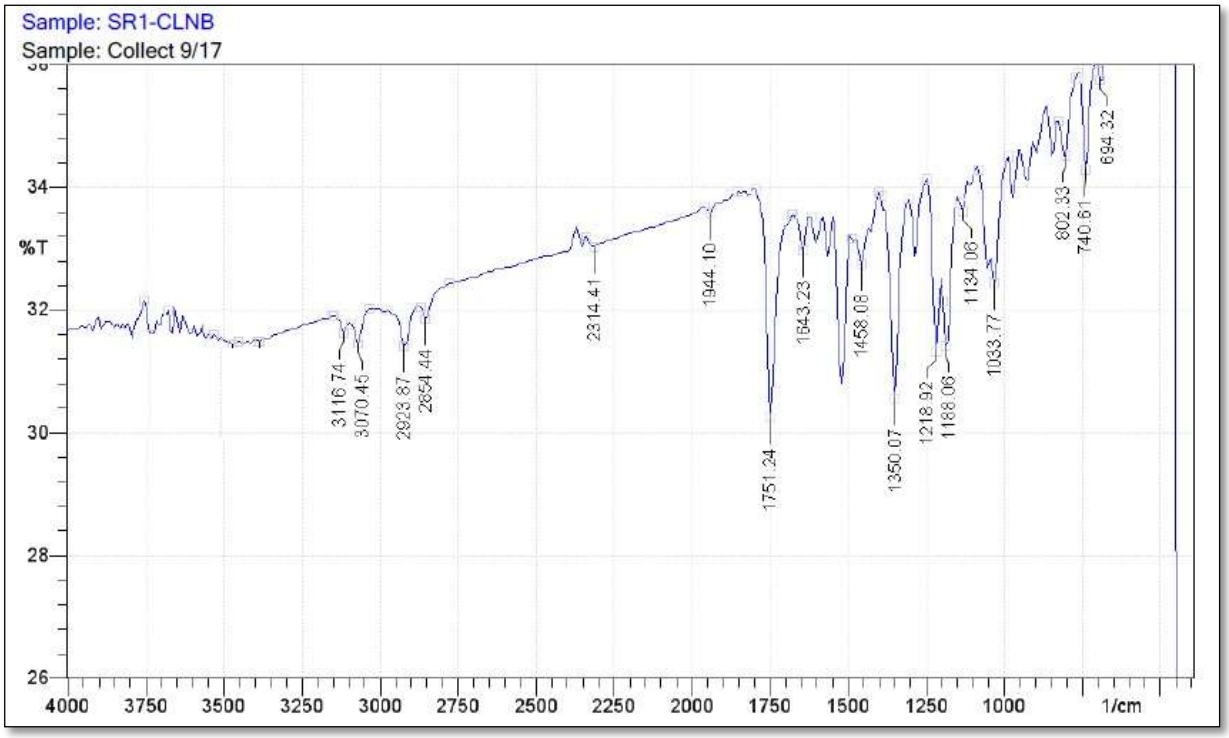


Fig 13: IR Spectra of SR 1

Compound	**IR Absorption region (cm^{-1})**	**Interpretation**
SR 2	3116 cm^{-1}	Aromatic C-H stretching
	3070 cm^{-1}	Alkene C-H stretching
	2923 cm^{-1}	Aliphatic C-H stretching
	1643 cm^{-1}	Aliphatic C=C stretching
	1751 cm^{-1}	Aliphatic ketone (C=O) stretching
	1458 cm^{-1}	Aromatic C=C stretching
	1350 cm^{-1}	Nitro N=O stretching

NMR SPECTROSCOPY

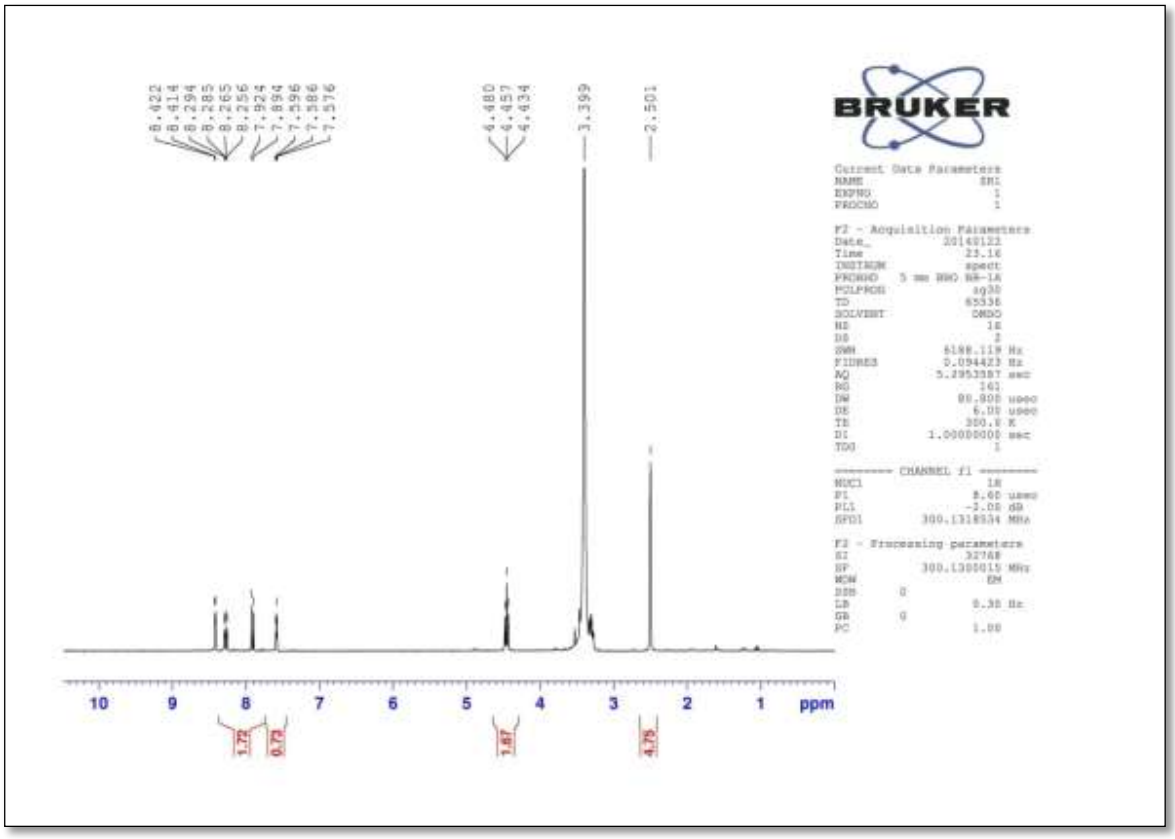


Fig 14: NMR Spectra of SR 1

Compound	**^{1}H NMR Data**
SR 1	δ 2.54-2.56 (m, 5H, Heterocyclic CH_2) δ 4.42-4.45 (d, 1H, Vinylic CH) δ 4.46-4.47 (d, 1H, Vinylic CH) δ 7.60-8.52 (m, 3H, Aromatic CH)

MASS SPECTROSCOPY

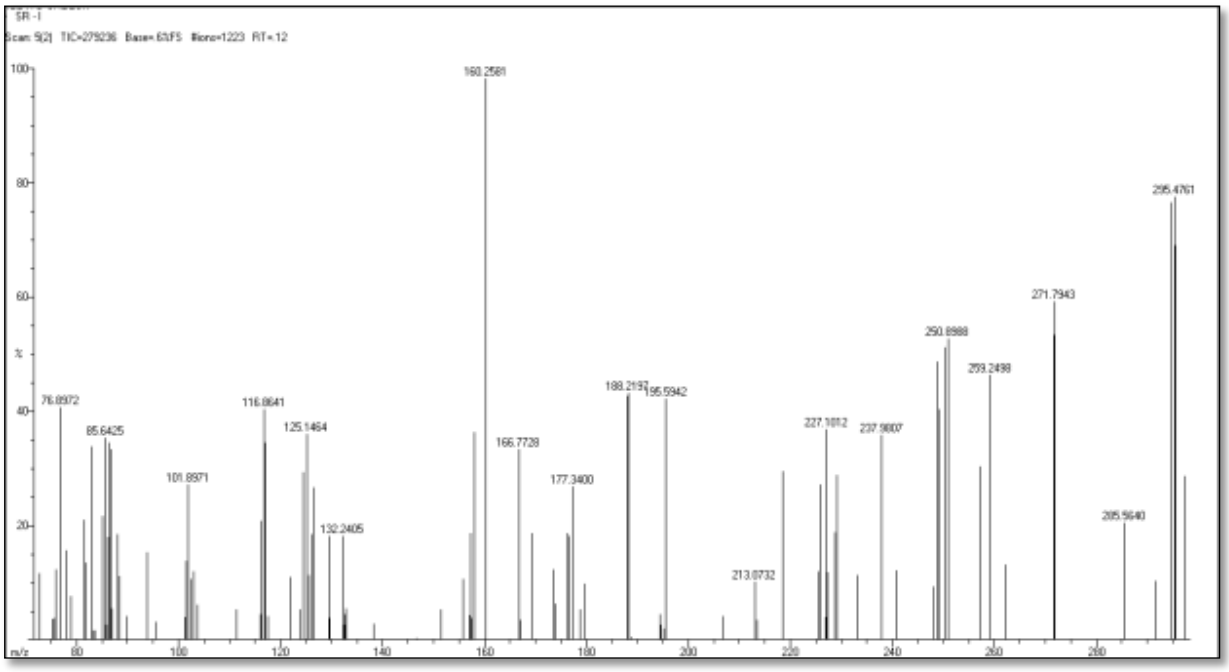
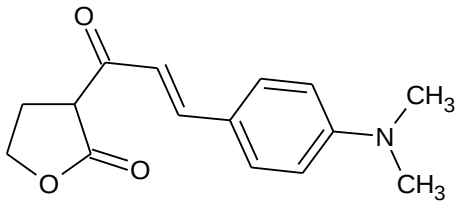


Fig 15: Mass Spectra of SR 1

Compound	**Mol. formula / Mol. Wt calculated**	**m/e value & Relative abundance**
SR 1	$C_{13}H_{10}ClNO_5$ /295.67	295.47 (M^+) 160.25 (B)

Analytical Data: Compound SR2



3-{(2*E*)-3-[4-(dimethylamino) phenyl] prop-2-enoyl}dihydrofuran-2(3*H*)-one

S. No	Properties	
1.	Description	Yellowish orange crystals
2.	Molecular Formula	$C_{15}H_{17}NO_3$
3.	Molecular Weight	259.30
4.	Melting Point	118 ºC
5.	Solubility	Soluble in ethanol and other organic solvents, insoluble in water

Table No 4: Physical properties of SR 2

IR SPECTROSCOPY

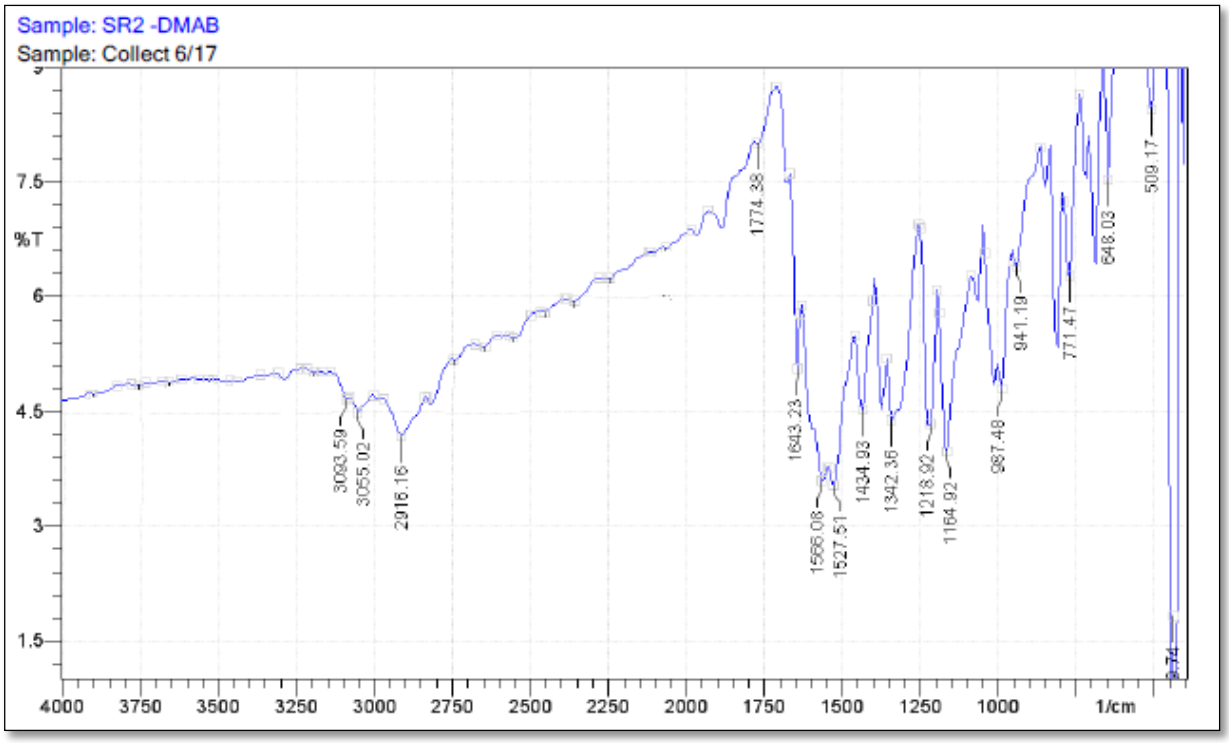


Fig 16: IR Spectra of SR 2

Compound	**IR Absorption region (cm^{-1})**	**Interpretation**
SR 2	3093 cm^{-1} 3055 cm^{-1} 2916 cm^{-1} 1774 cm^{-1} 1643 cm^{-1} 1434 cm^{-1}	Aromatic C-H stretching Alkene C-H stretching Aliphatic C-H stretching Aliphatic ketone (C=O) stretching Aliphatic C=C stretching Aromatic C=C stretching

NMR SPECTROSCOPY

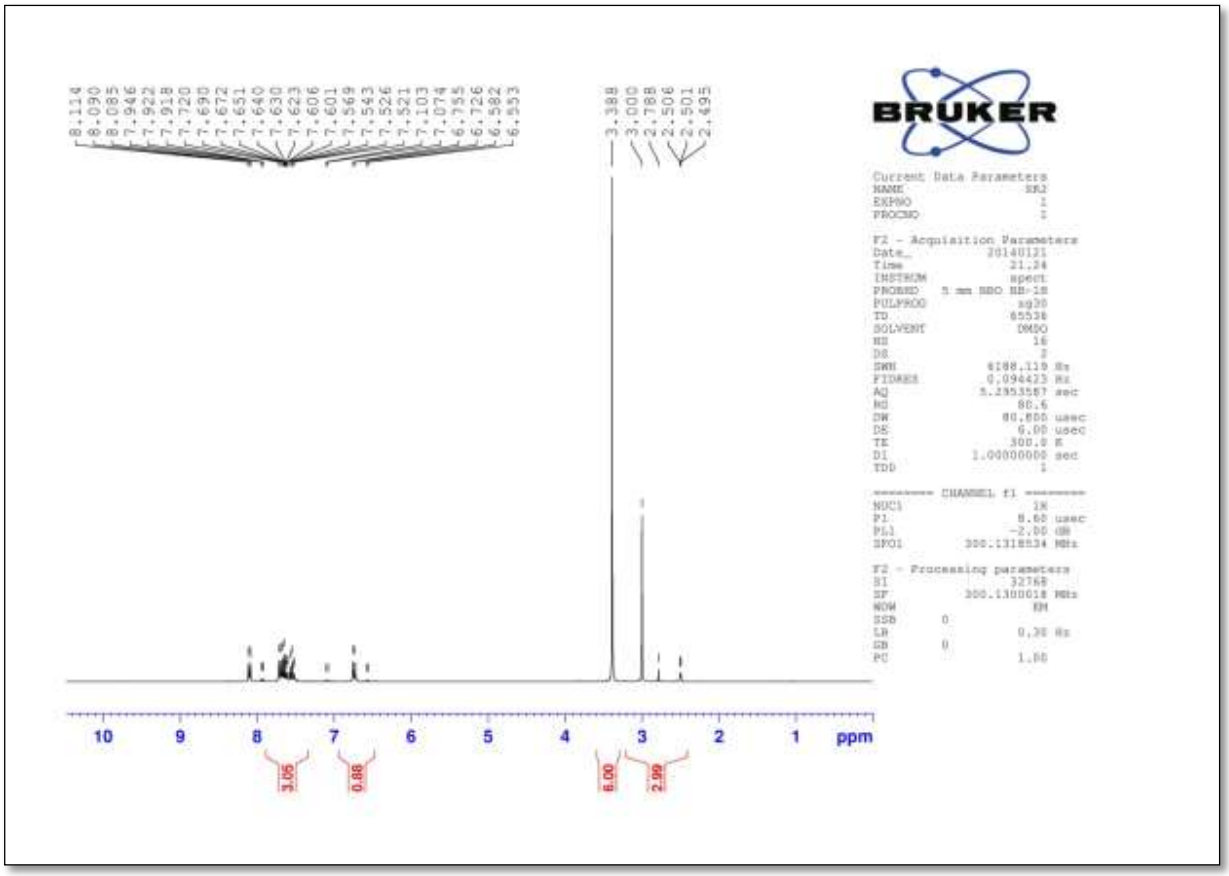


Fig 17: NMR Spectra of SR 2

Compound	**^{1}H NMR Data**
SR 2	δ 2.43-3.20 (m, 3H, Heterocyclic CH_2) δ 3.32-3.35 (s, 6H, Aliphatic CH_3) δ 6.59-6.98 (d, 1H, Vinylic CH) δ 7.41-7.95 (m, 3H, Aromatic CH)

MASS SPECTROSCOPY

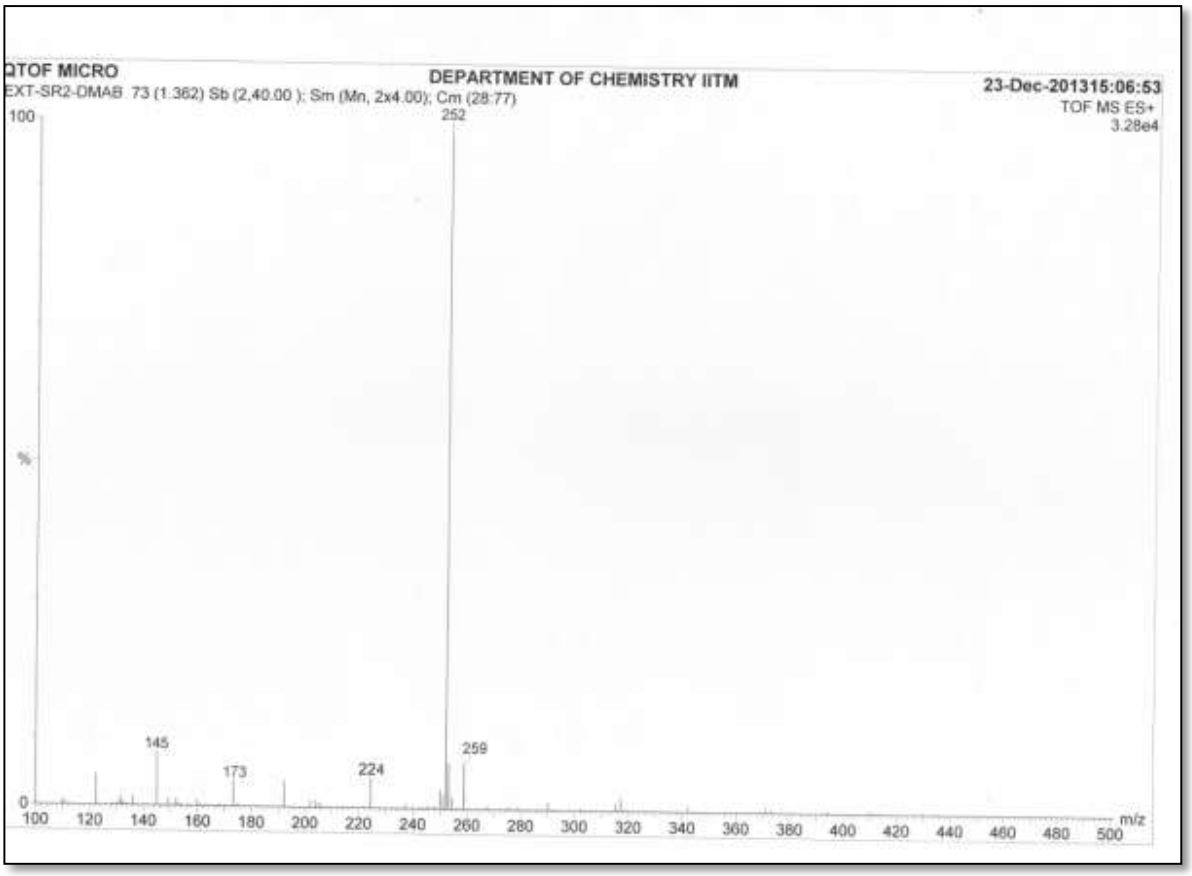


Fig 18: MASS Spectra of SR 2

Compound	**Mol. formula / Mol. Wt calculated**	**m/e value & Relative abundance**
SR 2	$C_{15}H_{17}NO_3$ / 259.30	259 (M^+) 252 (B)

2. IN VITRO ANTITUBERCULAR ACTIVITY

The anti tubercular activity of the synthesized compounds was determined by MABA method. The organism used is mycobacterium tuberculosis. The pathogen tested was susceptible to all the synthesized compounds and the MIC for all the compounds was found. The data pertaining to those observations are tabulated below and the growth of the organism is shown in the figure below. It was observed from the study that the MIC of all the synthesized compounds showed anti mycobacterial activity at 50 µg/ml and 100 µg/ml. The inhibition was compared with Pyrazinimide (3.125 µg/ml), Streptomycin (6.25 µg/ml), Ciprofloxacin (3.125 µg/ml).

Sl.No	Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1.	SR1	S	S	R	R	R	R	R	R
2.	SR2	S	R	R	R	R	R	R	R

Table No 6: Anti tubercular activity results in µg/ml.

NOTE:

S-Sensitive, R-Resistant

Strain Used: M.tuberculosis(H37 RV Strain)

Standard values:

Pyrazinimide- 3.125 µg/ml, Streptomycin-6.25 µg/ml, Ciprofloxacin- 3.125 µg/ml

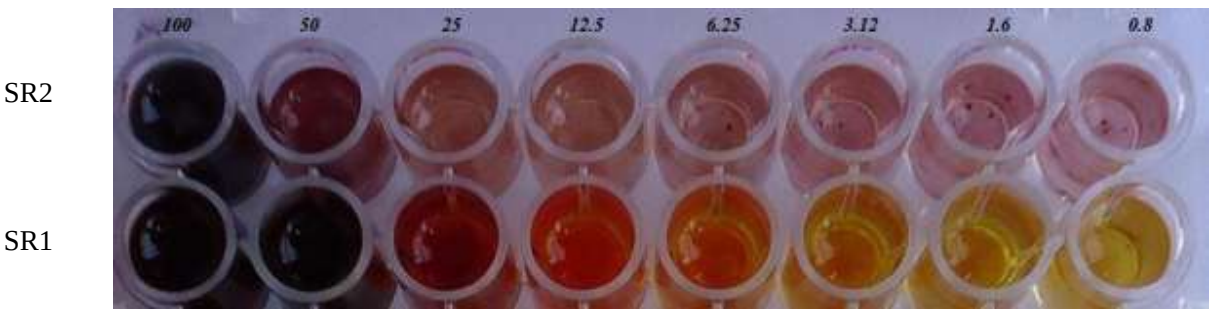


Fig 19: Invitro Antitubercular Activity by MABA

Summary and Conclusion

SUMMARY

- L, D Transpeptidase 2 is a crucial enzyme present in the cell wall of Mycobacterium tuberculosis H37Rv. It is a peptidoglycan binding protein. It plays a vital role in the cell wall synthesis.
- This enzyme was chosen as the target for the drug design study after detailed literature review.
- A database of 100 molecules with potential to inhibit the target (PDB id: 3VAE) was chosen by altering the lead butyrolactone nucleus.
- The designed molecules were docked against the chosen target using GLIDE® (Grid Based Ligand Docking with Energetics).
- From among the docked molecules, 2 molecules with good Glide score were chosen for further laboratory synthesis. Drug likeness was predicted insilico before proceeding for synthesis. The reaction conditions were optimized.
- The synthesized compounds were labeled as SR1 and SR2 and recrystallised.
- The purity of the synthesized compounds was assessed by melting point and TLC. The synthesized compounds were characterized by Infrared Spectroscopy, Nuclear Magnetic Resonance Spectroscopy and Mass Spectroscopy.
- The purified compounds were screened for antitubercular activity by *invitro* Micro Plate Alamar Blue Assay.
- The synthesized compounds SR1 and SR2 showed activity at 50 mcg/ml and 100 mcg/ml respectively. The MIC of known TB drugs Pyrazinamide: 3.125 mcg/ml, Ciprofloxacin: 3.125 mcg/ml and Streptomycin 6.25 mcg/ml.

CONCLUSION

- It is thus concluded that the synthesized compounds might effectively inhibit the chosen target, L, D Transpeptidase 2 which is crucial for the growth of Mycobacterium tuberculosis.
- These molecules can be further modified structurally and more prospective potential molecules against the *Mycobacterium tuberculosis* can be developed.

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